



~~Spisulosine compounds~~

Compositions and Methods Comprising Long-Chain,

Straight-Chain 2-Amino-3-Hydroxyalkanes

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation of copending application Serial No. 09/386,724, filed August 31, 1999, allowed as U.S. Pat. 6,800,661, which is a continuation-in-part of copending application Serial No. 09/058,456, filed April 10, 1998, allowed as U.S. Pat. 6,107,520, re-issued as U.S. RE38,793, which claims the benefit of U.S. Provisional Application Ser. No. 60/043,326, filed April 15, 1997, and U.S. Provisional Application Ser. No. 60/043,599, the disclosure of which is hereby incorporated herein by reference.

FIELD OF THE INVENTION

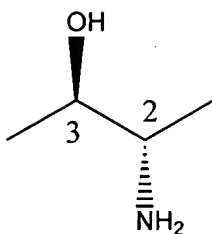
The present invention relates to pharmaceutical compositions of spisulosine compounds. It further relates to the treatment of ~~tumour~~tumors, and provides new cytotoxic compounds and pharmaceutical compositions for use against ~~tumour~~tumors. In one aspect, the invention relates to ~~antitumour~~antitumor compounds from marine organisms.

BACKGROUND OF THE INVENTION

There has been considerable interest in isolating bioactive compounds from marine organisms. Typical procedures involve *in vitro* screening programs to test crude extracts for antimicrobial, antiviral, and cytotoxic activities. Illustrative examples of known bioactive compounds from marine sources include bryostatins, ecteinascidins and furthermore didemnins where didemnin B, also now known as aplidine, is the first marine natural product in clinical testing.

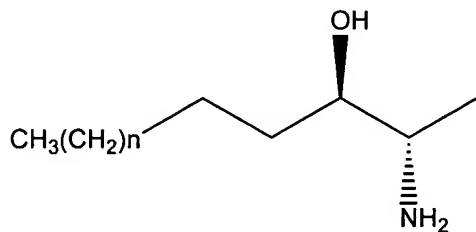
SUMMARY OF THE INVENTION

The present invention provides new pharmaceutical compositions containing a long-chain, straight-chain alkane or alkene compound which has a 2-amino group and a 3-hydroxy group, together with a pharmaceutically acceptable carrier. Typically the compound is a 2-amino-3-hydroxyalkane or a 2-amino-1,3-dihydroxyalkene. Preferably the compound is a substituted C₁₆-C₂₄ alkane or alkene. The compound is preferably a substituted alkane, more preferably a substituted C₁₈-C₂₀ alkane, and most preferably a 2-amino-3-hydroxy C₁₈ alkane. The substituted alkene is preferably a substituted mono- or di-alkene, more preferably a substituted C₁₈-C₂₀ alkene. In one embodiment, the compounds have the partial stereochemistry:

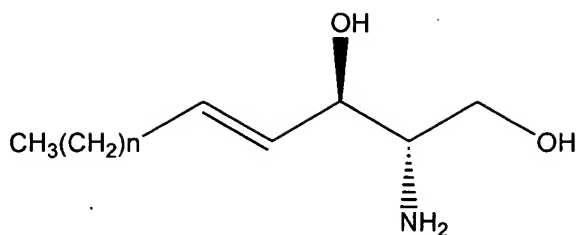


In particular, the present invention provides compositions which contain bioactive sphingoid-type bases, spisulosines 285, 299 and 313 (**1-3**), sphingosine (also referred to as 4-sphingenine or octadeca-4-sphingenine, **4**) and two related compounds, nonadeca-4-sphingenine (a one carbon longer homologue, **5**) and sphinga-4,10-diene (a dehydrosphingosine derivative, **6**).

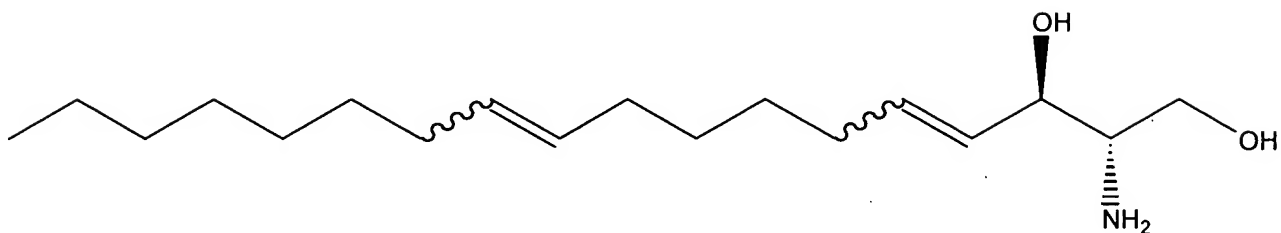
Thus, the preferred compositions contain one or more of the following preferred compounds:



spisulosine 285 (**1**), n=12; spisulosine 299 (**2**), n=13; spisulosine 313 (**3**), n=14;



as well as sphingosine (4), $n=12$ and nonadeca-4-sphingenine (5), $n=13$; and



spinga-4,10-diene (6).

The preferred compound, spisulosine 285, is known in the literature. Compound 1 and the *syn* diastereoisomer, were first synthesized by Croatian researchers in the determination of absolute configurations of lipid bases with two or more asymmetric carbon atoms, see Proštenik, M., Alaupovic, P. *Croat. Chem. Acta*. 1957, 29, 393.

It is believed that the other compounds in the compositions of this invention are new compounds.

Compounds **1-3** show unique cytotoxicity against L1210 murine lymphocytic leukemia cells. In a number of the L1210 assays, a distinct morphological alteration was observed. This effect was also described in our earlier U.S. Provisional Patent Application Serial No. 60/043,326. We make no patent claim in this patent application to the effect itself on L1210, and indeed there is now some preliminary data ~~which~~that suggests that the compounds such as spisulosine 285 might lack activity against leukemia ~~tumour~~tumors.

A synthetic sample of **1** was assayed against L1210 leukemia cells and showed both cytotoxicity and morphological alteration, pointed cell activity.

L1210 Inhibition and pointed cell activity

Concentration	% cytotoxicity	% pointed cells ^a
0.5 µg/ml	100	97
0.25 µg/ml	99	100
0.1 µg/ml	99	62
0.05 µg/ml	96	71
0.025 µg/ml	90	21
0.01 µg/ml	45	1

^a Percent pointed cells are a percent of the living cells.

Spisulosine 285 (**1**) is also active against other ~~tumour~~tumor cell lines *in vitro*, including P-388 (0.01 mg/ml); A-549 (0.05 mg/ml); HT-29 (0.05 mg/ml) and MEL-28 (0.05 mg/ml).

In a particularly preferred embodiment, the present invention relates to use of spisulosine 285, and related compounds, in the treatment of all types of cancer, such as breast cancers, prostate, bladder, pancreas, lung, ~~oesophagus~~esophagus, larynx, liver, colon, thyroid, melanoma, kidney, testicular, ~~leukaemia~~leukemia, ovarian, gastro-intestinal, hepatocellular carcinoma and vascular endothelial cancer. Other forms of cancer are well known to the person skilled in the art. It is preferred that the use of spisulosine 285, and related compounds is against solid ~~tumour~~tumors, with use against slow proliferating ~~tumour~~tumors such as prostate, lung, liver, kidney, endocrine gland and vascular endothelial cancer particularly preferred. In one aspect, the compositions are for use in therapy directed at the vascular endothelium for control of tissue and ~~tumour~~tumor vascularisation.

The present invention is directed to bioactive compounds that have been found to possess specific ~~antitumour~~antitumor activities and as such they will be useful as medicinal agents in mammals, particularly in humans. Thus, another aspect of the present invention concerns pharmaceutical compositions containing the active compounds identified herein and methods of treatment employing such pharmaceutical compositions.

The active compounds of the present invention exhibit ~~antitumour~~antitumor activity. Thus, the present invention also provides a method of treating any mammal affected by a malignant ~~tumour~~tumor sensitive to these compounds, which comprises administering to the affected individual a therapeutically effective amount of an active compound or mixture of compounds, or pharmaceutical compositions thereof. The present invention also relates to pharmaceutical preparations, which contain as active ingredient one or more of the compounds

of this invention, as well as the processes for its preparation.

Examples of pharmaceutical compositions include any solid (tablets, pills, capsules, granules, etc.) or liquid (solutions, suspensions or emulsions) with suitable composition or oral, topical or parenteral administration, and they may contain the pure compound or in combination with any carrier or other pharmacologically active compounds. These compositions may need to be sterile when administered parenterally.

Administration of the composition of the present invention may be by any suitable method, such as intravenous infusion, oral preparations, intraperitoneal and intravenous administration. Intravenous delivery may be carried out over any suitable time period, such as 1 to 4 hours or even longer if required, at suitable intervals of say 2 to 4 weeks. Pharmaceutical compositions containing spiculoline may be delivered by liposome or nanosphere encapsulation, in sustained release formulations or by other standard delivery means.

The correct dosage of a pharmaceutical composition comprising the compounds of this invention will vary according to the particular formulation, the mode of application, and the particular situs, host and bacteria or ~~tumour~~tumor being treated. Other factors like age, body weight, sex, diet, time of administration, rate of excretion, condition of the host, drug combinations, reaction sensitivities and severity of the disease shall be taken into account. Administration can be carried out continuously or periodically within the maximum tolerated dose.

The compounds may be provided in the pharmaceutical compositions of this invention in the form of a prodrug or precursor, which upon administration converts or is ~~metabolised~~metabolized to the active compound.

The compositions of this invention may be used with other drugs to provide a combination therapy. The other drugs may form part of the same composition, or be provided as a separate composition for administration at the same time or a different time. The identity of the other drug is not particularly limited, and suitable candidates include:

- a) drugs with antimitotic effects, especially those which target cytoskeletal elements, including microtubule modulators such as taxane drugs (such as taxol, paclitaxel, taxotere, docetaxel), podophylotoxins or vinca alkaloids (vincristine, vinblastine);
- b) antimetabolite drugs such as 5-fluorouracil, cytarabine, gemcitabine, purine analogues such as pentostatin, methotrexate);
- c) alkylating agents such as nitrogen mustards (such as cyclophosphamide or ifosfamide);
- d) drugs which target DNA such as the anthracycline drugs adriamycin, doxorubicin, pharmorubicin or epirubicin;
- e) drugs which target topoisomerases such as etoposide;

f) hormones and hormone agonists or antagonists such as estrogens, antiestrogens (tamoxifen and related compounds) and androgens, flutamide, leuprorelin, goserelin, cyprotrone or octreotide;

g) drugs which target signal transduction in tumour cells including antibody derivatives such as herceptin;

h) alkylating drugs such as platinum drugs (cis-platin, carboplatin, oxaliplatin , paraplatin) or nitrosoureas;

i) drugs potentially affecting metastasis of tumours such as matrix metalloproteinase inhibitors;

j) gene therapy and antisense agents;

k) antibody therapeutics; and

l) other bioactive compounds of marine origin, notably the ecteinascidins such as ET-743, or the didemnins such as aplidine.

The present invention also extends to the compounds for use in a method of treatment, and to the use of the compounds in the preparation of a composition for treatment of cancer.

Spisulosine 285 has an effect upon cell morphology. Vero cells treated with spisulosine 285 had a reduced microfilament structure, as assessed by staining of the spisulosine-treated cells with phalloidin, which stains actin in the microfilaments. Spisulosine 285 also affects the distribution of the small GTP binding protein Rho, although this effect may be reduced or eliminated by pre-treatment with the Rho-activator LPA (Mackay and Hall, *J. Biol. Chem.*, 273, 20685-20688, 1998).

Without wishing to be constrained by theory, we believe that the mechanism of action of spisulosine 285 may involve modulation of the action of the small GTP binding protein Rho, possibly via an effect on LPA activation. Rho is known to be involved in the formation of stress ~~fibres~~fibers (Hall, A., *Science* 279, 509-514, 1998), and has a role in controlling cell adhesion and motility through ~~reorganisation~~reorganization of the actin cytoskeleton (Itoh, et al, *Nature Medicine*, Vol 5, No. 2, 1999). Adhesion of ~~tumour~~tumor cells to host cell layers and subsequent transcellular migration are key steps in cancer invasion and metastasis. By affecting (reducing) the levels of microfilaments in the cell, via an (inhibitory) effect on Rho, spisulosine 285 may serve to limit the development of cancer via an effect on the cell cytoskeleton. It is also known that Rho triggers progression of the G1 phase of the cell cycle. As such, modulation of Rho may also prevent cellular transformation by stopping cell cycle progression. Therefore, the present invention also relates to the use of spisulosine compounds in the preparation of a medicament for the treatment of cancer, wherein the spisulosine compound acts to alter Rho protein activity.

LPA, an activator of Rho, can help prevent the effect of spisulosine compounds on microfilament formation. ~~Whilst~~While the specific target of spisulosine 285 is not known, the

observed reduction of actin microfilaments in cells treated with spisulosine 285 and the lipid structure of spisulosine 285 suggest that spisulosine compounds may serve as an antagonist for the LPA receptor, preventing LPA interacting with its receptor to activate Rho to produce the microfilaments.

The preferred compounds of this invention were initially isolated from *Spisula polynyma*. *Spisula polynyma* is an edible clam, which is also known as the Stimpson surf clam or the Atlantic surf clam. It belongs to the subfamily Mactrinae, family Mactridae, superfamily Mactroidea, order Veneroida, subclass Heterodonta, class Bivalvia, phylum Mollusca. *Spisula polynyma* was originally found off the coast of Japan, where it is called hokkigai and processed for sushi. It has now migrated through the Bering Strait, down past Greenland and Newfoundland, into the Atlantic ocean. The clam has a grey-white shell, 7-10 cm long. It is mainly off-white, except for the tongue which is purple in the living clam, but turns bright red after cooking.

Thus, the present invention provides active extracts of the clam *Spisula polynyma*. One embodiment of the present invention is directed to novel compounds isolated from the clam *Spisula polynyma*, and the use of all of the cytotoxic compounds isolated therefrom as ~~antitumor~~antitumor compounds.

To test for biological activity, one clam was homogenized in 3:1 methanol/toluene. A solution of sodium chloride was added to this crude extract, causing it to separate into a toluene and an aqueous layer. The latter was further extracted with toluene, dichloromethane, ethyl

acetate and 1-butanol. These extracts were all assayed against L1210 cells, where significant cytotoxicity was observed for the initial crude, toluene and dichloromethane extracts and less activity in the other three fractions.

L1210 Cytotoxicity of Crude Extracts of *Spisula polynyma*^{a,b}

	Concentration (µg/ml)					
Extract	250	125	50	25	12.5	5
Crude	98*	98*	92	25	0	0
Toluene	100*	100*	100*	25	13	13
CH ₂ Cl ₂	100*	100*	100*	91	20	13
EtOAc	98*	98*	92*	0	0	0
1-BuOH	83	33	0	0	0	0
Aqueous ^c	94	75	0	0	0	0

Footnotes:

(a) cytotoxicity reported as % inhibition of growth;

(b) entries marked with * showed pointed cell activity;

(c) the aqueous extract was assayed at 700, 350, 140, 70, 35 and 14 mg/ml.

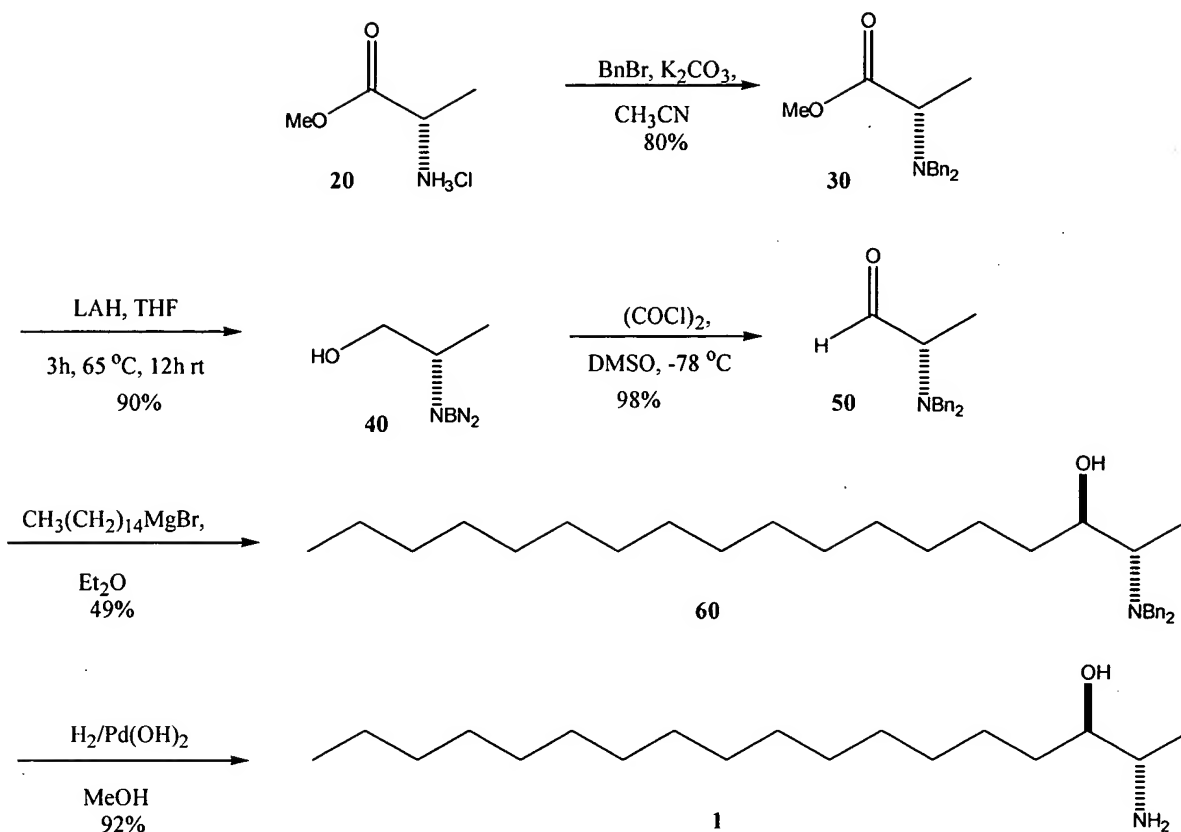
These extracts were also assayed against *Herpes simplex* virus Type 1 (HSV-1) and CV-1 monkey kidney cells (at 100 mg/6.35-mm disk), but no activity was observed. No antimicrobial activity was observed for these extracts against *Penicillium melinii* (formerly *P. atrovenetum*) and *Micrococcus luteus* (formerly *Sarcina lutea*), both at 500 mg/ 12.7- mm-disk). Later, other

more purified extracts were assayed against *Bacillus subtilis*, *Saccharomyces cerevisiae*, and *Escherichia coli* with no bioactivity observed.

Synthetic methods are also available for the preparation of spisulosine compounds, particularly spisulosines 285 (1), 299 (2) and 313 (3).

The preferred synthetic route is based upon the previous addition of organometallics to N,N-dibenzylamino aldehydes to yield ~~α -amino~~ β -amino alcohols with high stereoselectivity. See, Andres et al., *Org. Chem.* 1996, 61, 4210 and Reetz et al., *Angew Chem. Int. Ed. Engl.*, 1987, 26, 1141. The non-chelation controlled addition of Grignard reagents or organolithium compounds produces the *anti*-diastereomer and the chelation controlled addition of organozinc preferentially gives the *syn*-diastereomer.

Scheme I illustrates this preferred synthetic process for the formation of Compound 1:



As described in Scheme 1, the β -amino aldehyde **50** can be prepared from L-alanine methyl ester by first dibenylation of the amino group with benzyl bromide and potassium carbonate followed by lithium aluminum hydride reduction to the N,N-dibenzylamino alcohol **40**. The Swern oxidation of **40** gives **50** in high yield and can be used without further purification to avoid decomposition. Addition of the Grignard reagent to **50** gives the *anti*-diastereomer **60** with high selectivity. The compound, **60**, can be easily purified, for example by flash chromatography and HPLC. The deprotection of **60** by hydrogenolysis on Pearlman's catalyst gives **1** in high yield and a good overall yield. Compounds **2** and **3** may be prepared simply by

increasing the chain length of the Grignard reagent, and the remaining compounds of the present invention may also be prepared by appropriate choice of the Grignard reagent.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A, 1B, 1C, 1D, 1E and 1F are illustrations of the cell morphologies observed in the L1210 assays of *Spisula polynyma* extracts. Figure 1A represents a normal cell; Figure 1B represents a typical pointed cell; Figure 1C represents an atypical pointed cell; Figure 1D represents a cell with more than two points; Figure 1E represents a bulged cell; and Figure 1F represents a combined bulged and pointed cell.

Figure 2 illustrates the scheme used to separate the compounds described herein from extracts of the clam *Spisula polynyma*.

Figure 3 is a microphotograph for the results in Example A.

Figure 4 is a microphotograph for the results in Example B.

Figure 5 is an electrophoretogram of Example C.

Figure 6 is a microphotograph for the results in Example D.

DETAILED DESCRIPTION OF THE INVENTION

Referring to Figure 1, in the L1210 assay some of the cells changed from being spherical (Figure 1A) to ovoid with long points approximately 180 °C apart (Figure 1B). Several other forms have also been observed in assays of these extracts, including cells with points not 180 °C apart (Figure 1C), cells with more than two points (Figure 1D), cells with a bulge (Figure 1E) and cells with a bulge replacing one of the points (Figure 1F). However, the form with two sharp, opposing points was by far the predominant and characteristic one observed. This type of morphological change had not previously been observed during the screening of over 1000 marine extracts.

Isolation of Spisulosines 285, 299, and 313

For this invention, *Spisula polynyma* were collected, at a depth of -110 feet, from a clam bed on the eastern edge of Stellwagon bank which is located off the coast of New England, stretching from near Gloucester, Mass., north to Maine. They were shipped live by the New England Clam Corporation (formerly New Dawn Seafoods, Inc.) and then immediately frozen.

A purification scheme similar to the extraction procedure described above for the original testing of the bioactivity was employed. First 35 clams were thawed and the shells removed to give 1.9 kg (wet wt). These were allowed to stand in 3:1 methanol/toluene and filtered after several hours. This step was repeated followed by homogenization and extensive extraction with this same solvent to give a crude extract. To this was added a 1 M sodium chloride solution

which caused the extract to separate into two layers. The lower aqueous layer was further extracted with toluene and the toluene layers combined. The resulting aqueous layer was then extracted with dichloromethane as shown in Figure 2.

The toluene extract was partitioned between methanol and hexane. The cytotoxicity and cellular alteration were observed almost exclusively in the methanol fraction. The methanol extract thus obtained was applied to a silica flash column, eluting with a chloroform/methanol step gradient (100:0, 99:1, 95:5, 90:10, 85:15, 80:20, 70:30, 50:50, 0:100). The main cytotoxic and pointed-cell-forming activity eluted off the column very late, although earlier fractions did show some cytotoxicity, but no pointed cells. This late eluting was further purified by flash silica chromatography, using 8:12:1:1 chloroform/1-butanol/acetic acid/water. Fractions were neutralized with sodium bicarbonate before removing the solvent to prevent possible decomposition when they were concentrated in acid. This resulted in a series of three bioactive fractions.

It had been observed in earlier attempts at isolation that the bioactivity did not wash off of a cyano solid-phase extraction (SPE) column with methanol, but the cytotoxicity was found to elute with 3:1 methanol/0.01 M ammonium formate (0.5 ml/min). This was confirmed by chromatographing a small amount of a bioactive fraction on a cyano HPLC column with this same solvent system and then repeating the injection under the same conditions except replacing the ammonium formate solution with water. The chromatograms appeared identical except that a peak eluting at 15.6 min was only observed in the first.

The three bioactive fractions from the second silica column were each further purified by cyano HPLC with the same conditions used above (except 1 ml/min) to give three series of bioactive fractions. The ammonium formate was removed by passing the sample through a C-18 SPE column, washing first with water and then eluting with methanol. The main cytotoxicity and morphology-changing activity of each series (fractions A, B, and C) was found in a peak comparable to that discussed above. However the activity was spread throughout most of the fractions. Silica TLC (3:12:2:2 chloroform/1-butanol/acetic acid/water) indicated that fraction A (0.4 mg) contained one spot (R_f 0.47), which was pink by ninhydrin. Fraction B (1.3 mg) showed this same spot as well as one slightly lower (R_f 0.44, red by ninhydrin), while fraction C (0.2 mg) contained both of these and a third one (R_f 0.34, purple by ninhydrin). All three showed good cytotoxicity and pointed-cell forming activity, with A exhibiting slightly more activity than B and significantly more than C. This indicated that the uppermost TLC spot must be from compound(s) which caused the morphological change in L1210 cells. These fractions were not purified further, but analyzed as mixtures. Quantitative bioassay results are discussed below.

An attempt was made to determine if a particular organ of *Spisula polynyma* contained most or all of the bioactivity. A live clam was anesthetized with diethyl ether and then dissected into nine parts: foot, digestive system, gonads, siphon, gills, heart, mantle, adductor muscles, and the remainder of the visceral mass (with foot, digestive system and gonads removed). These were identified by comparison to illustrations of other clams. Each organ was homogenized in 3:1 methanol/toluene and the resulting extract was then triturated with dichloromethane and methanol to remove salts. While all of the extracts showed cytotoxicity (Table), only those from the gills and the gonads exhibited strong morphology-changing activity. That from the digestive

system and the remainder of the visceral mass also showed weak pointed-cell forming activity, possibly due to incomplete separation from the gonads. The lack of pointed-cell-forming activity in other organs may have resulted either from a lack of 1-3 or from a much lower concentration.

In another experiment, one foot that had been cooked for a brief period was extracted in an analogous manner. This also showed cytotoxicity, but no morphology-altering activity. However, when a larger sample of cooked material was more extensively extracted, some pointed cells were observed in the L 1210 assay. Silica TLC (3:12:2:2 chloroform/1-butanol/acetic acid/water, 100 mg) of the extracts of the digestive system and gonads showed a weak ninhydrin-positive spot at R_f 0.49.

Organ	250 µg/ml		125 µg/ml		50 µg/ml	
	%	%	%	%	%	%
	Inhibition	Pointed ^a	Inhibition	Pointed ^a	Inhibition	Pointed ^a
foot	100	0, ad ^b	100	0,ad	93	0,0
digestive system	96	0,18	62	0,0	0	0,0
gonads	nr ^c	nr	99	56,100	90	32,100
siphon	100	ad,ad	50	0,0	0	0,0
gills	100	ad,ad	100	50,ad	98	100,93
heart	100	ad,ad	nr	0,0	38	0,0
mantle	100	0,0	99	0,0	95	0,0
adductor	100	added	100	0,0	95	0,0

muscles

visceral	100	10,ad	100	2,ad	94	0,0
----------	-----	-------	-----	------	----	-----

mass

cooked	100	0	100	0	97	0
--------	-----	---	-----	---	----	---

foot^d

cooked	91	21	25	0	0	0
--------	----	----	----	---	---	---

foot^e

footnotes:

^aPercentage of pointed cells was measured at 58 to 82 h after the start of the assay.

^bad = all dead.

^cnr = not read due to precipitated material in the assay which obscured the cells.

^dPercentage of pointed cells measured at 72 h after the start of the assay.

^eThis sample was extracted in a similar manner to that used to obtain the crude extract of the isolation of fractions A-C. Percentage of pointed cells measured at 76 h after the start of the assay.

Several clues to the structure of the bioactive compounds could be found in the isolation procedure. The TLC spot which correlated with the activity visualized as pink or red by ninhydrin, suggesting that the compounds contained primary amines. Also, they exhibited amphiphilic character. They were originally extracted into toluene from aqueous methanol, but they then partitioned into methanol versus hexane. While they are soluble in nonpolar solvents, they require a very polar solvent (3:12:2:2 chloroform/1-butanol/acetic acid/water) to be eluted

from silica.

Only fractions A and B were reasonably pure from inactive contaminants as shown by TLC. Most of the structure determination studies were carried out on fraction B because of its size relative to the others. Figures 3 and 4 show the ^1H NMR spectra of this fraction in CDCl_3 and CD_3OD , respectively. What was immediately obvious in these spectra was a peak corresponding to a long methylene chain (1.25 ppm) and several overlapping terminal methyl groups (0.87 ppm). Other peaks were not as well defined. No peaks corresponding to aromatic protons were observed, but several peaks appeared in the alkene proton region. Several others seemed to correspond to protons attached to heteroatom-substituted carbons. The major difference between the spectra in the two different solvents was that, in CD_3OD , a methyl doublet (1.21 ppm) downfield of the terminal methyl groups were clearly observed, while in CDCl_3 this resonance appeared only as an upfield shoulder on the methylene chain peak.

An authentic sample of *D-trans-erythro*-sphingosine (**4**) was obtained from Sigma for comparison with the isolated material. The ^1H NMR spectrum thereof was similar in many respects to that of fraction B. As expected, **4** exhibited a large peak due to the long methylene chain (1.25 ppm), a terminal methyl group (0.87 ppm) and two vinyl protons (5.75 and 5.46 ppm). Of particular note was the broadness of the resonances corresponding to protons on the heteroatom-substituted carbons (4.40, 3.66, 2.85 and 2.18 ppm). Also, on silica TLC (3:12:2:2 chloroform/1-butanol/acetic acid/water), **4** had R_f 0.43 and appeared red by ninhydrin, like the lower spot in fraction B and the middle spot in C. Palmeta and Proštenik have reported that 2-amino-3-octadecanol and **4** exhibited very similar R_f values (0.32 and 0.29, respectively) when

eluted on paper impregnated with silicic acid with the solvent system di-isobutyl ketone/acetic acid/water (40:25:5).

Fractions A-C were also studied by several mass spectrometric methods. The largest ion in all of the spectra was m/z 286. High resolution measurement of this peak (m/z 286.3109) allowed the assignment of the molecular formula $C_{18}H_{40}NO$ (Δ 0.1 mmu) to spisulosine 285 (**1**). This compound derived its name, in part, from its molecular weight. This molecular formula indicated that the molecule is totally saturated. A strong peak corresponding to the loss of water from this $M+H$ ion was observed at 268.3019 (Δ -1.5 mmu). Thus, **1** must contain a hydroxyl group. Ions corresponding to matrix adducts, of m/z 286 were observed at m/z 438.3078 ($C_{22}H_{48}NO_3S_2$, Δ -0.2 mmu), 590, and 592.

One well-known primary metabolite that, like **1**, consists of an 18 carbon chain substituted with hydroxyl and amine functionalities is sphingosine (**4**). This compound has one more oxygen and two less hydrogens than **1**. The analogy appeared valid because high resolution measurement of m/z 300 for the spisulosines indicated that it was a doublet corresponding to the $M+H$ of a higher homologue (**2**) of m/z 286 (300.3270, $C_{19}H_{42}NO$, Δ -0.4 mmu), together with sphingosine (**4**) itself (300.2914, $C_{18}H_{38}NO_2$, Δ -1.1 mmu). This also helped to explain the presence of alkene protons in the 1H NMR spectrum.

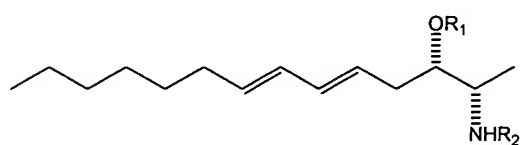
Several other peaks were evident in all three spectra. The ion at m/z 314 was also a doublet corresponding to $C_{20}H_{44}NO$ (314.3439, Δ -1.6 mmu), which was the molecular ion of another homologue of **1**, spisulosine 313 (**3**), and $C_{19}H_{40}NO_2$ (314.3075, Δ -1.6 mmu) which

was a homologue of sphingosine (**5**). Compound **4** showed matrix adducts of the M+H ion at m/z 452.2885 ($C_{22}H_{46}NO_4S_2$, Δ -1.7 mmu), 604.2831 ($C_{26}H_{54}NO_6S_4$, Δ 0.3 mmu) and 606.2995 ($C_{26}H_{56}NO_6S_4$, Δ 3.6 mmu), **5** exhibited matrix adducts of the M+H ion at m/z 464.2888 ($C_{23}H_{46}NO_4S_2$, Δ -2.0 mmu) and 618.2940 ($C_{27}H_{56}NO_6S_4$, Δ 5.1 mmu). It should be noted that, while m/z 300 and 314 were doublets of nearly equal intensity in fraction B, only one peak was measurable for the matrix adducts listed here from fraction B. This suggested that these two series of compounds, although very similar in general structure, behaved differently in FABMS. The spisulosine series (saturated) gave strong molecular ions and weaker matrix adducts, while the reverse was observed for the sphingosine series (unsaturated).

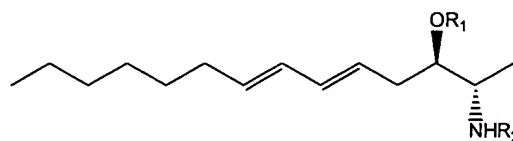
To better establish the structures identified by the data discussed above, several derivatives were prepared. The most informative was the diacetyl derivative of spisulosine **285** (**8**). Because fraction B was the largest, a portion of it was acetylated with acetic anhydride in pyridine. This mixture of acetyl derivatives will be referred to here as AcB. By silica TLC (3:12:2:2 chloroform/1-butanol/acetic acid/water), the reaction appeared quantitative, with a new spot appearing at R_f 0.86. For comparison, the triacetyl derivative of authentic **4** (**9**) was also synthesized by the same method.

Two series of compounds related to the spisulosines have been previously isolated. Gulavita and Scheuer reported that a *Xestospongia* sp. sponge from Papua-New Guinea contained two epimeric 14-carbon amino alcohols **134** and **135**. These were not isolated as the free amines, but rather the mixture was acetylated to give both the mono- (**136**, **137**) and diacetyl compounds (**138**, **139**) which were then separated. Jimenez and Crews have isolated several

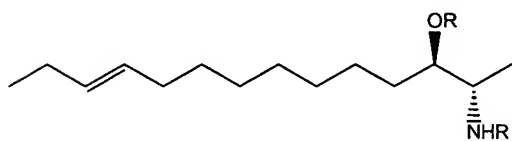
molecular ion of the underivatized **1** at m/z 286. This M+H ion (m/z 370) fragmented to give m/z 310 and 268, presumably by losing acetic acid and then the second acetyl group, respectively. The comparable ions for the other spingosines were small, but present: m/z 384, 324 and 282 for the diacetyl derivative of **2** (**144**), and m/z 398, 338 and 296 for the diacetyl derivative of **3** (**145**). The ions from the sphingosine in the sample were too small to state definitively that they were present. This again showed that the two series of compounds had very different ionization potentials. The CIMS spectrum showed strong m/z 370 and 310 ions, but here the m/z 268 ion was very weak. The higher homologues were again seen at m/z 384 and 324 for **144**, and m/z 398 and 338 for **145**. Weak ions at m/z 426 and 366 were indicative of **133**.



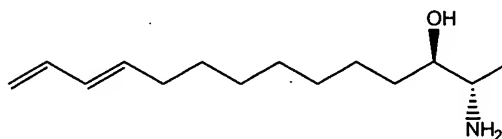
134 $R_1 = R_2 = H$
136 $R_1 = H, R_2 = Ac$
138 $R_1 = R_2 = Ac$



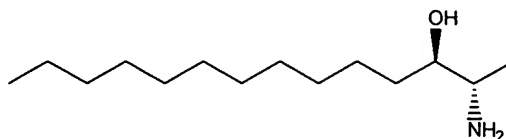
135 $R_1 = R_2 = H$
137 $R_1 = H, R_2 = Ac$
139 $R_1 = R_2 = Ac$



140 $R = H$
143 $R = Ac$



141

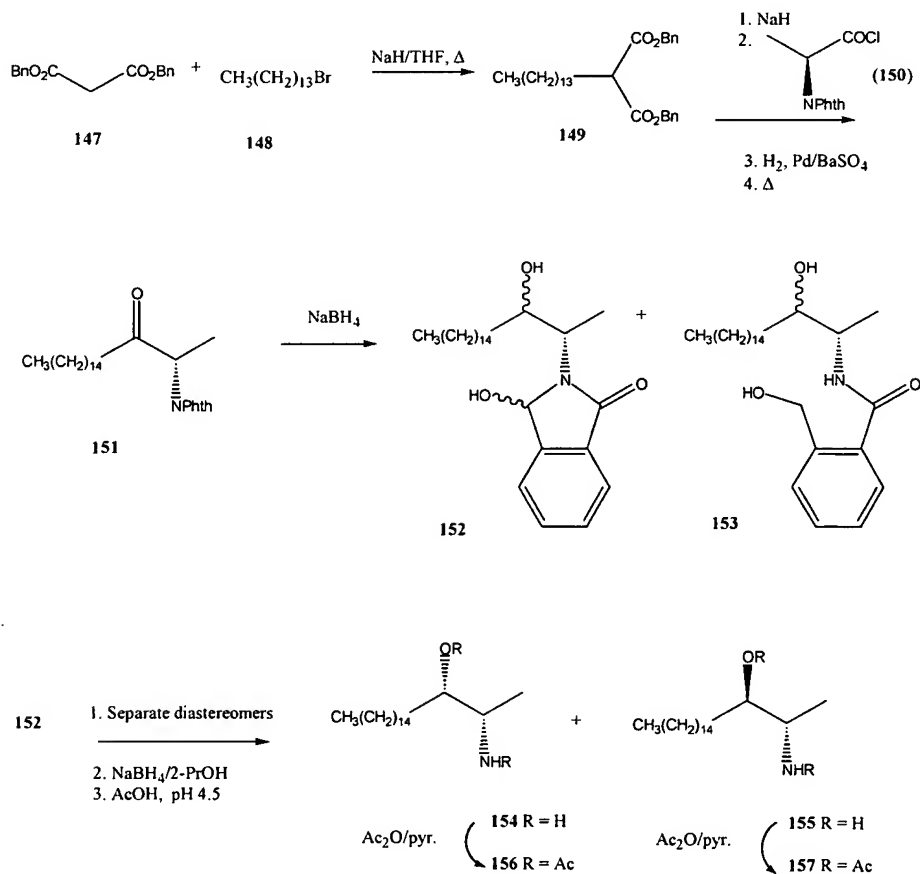


Synthesis of Spisulosine 285

To confirm the structure and determine the stereochemistry of spisulosine 285 the compound was synthesized. None of the isomers of 2-amino-3-octadecanol were previously known as natural products, but both the 2*S*, 3*S* and 2*S*, 3*R* isomers have previously been synthesized. The higher homologues are novel compounds.

A modified version of the synthesis of Proštenik and Alaupovic (Scheme IX) was used to obtain the authentic material for comparison.

Scheme IX:



First, dibenzyl malonate (**147**) was alkylated with tetradecyl bromide (**148**). The resulting dibenzyl tetradecylmalonate (**149**) was then condensed with N-phthaloyl-L-alanyl chloride (**150**) to give 2-phthalimido-3-octadecanone (**151**) after removal of the benzyl groups and decarboxylation. This ketone was treated with excess sodium borohydride, which resulted in the reduction of one of the phthalimido carbonyls in addition to the ketone, producing both **152**, which had one phthalimido carbonyl reduced to the carbinolamine, and **153**, which was further reduced. These two products could be readily separated from each other by silica flash chromatography.

The reduction of **151** to **152** produced a mixture of four diastereomers because of the formation of two new chiral centers. At this point, the diastereomers were separated by cyano HPLC. The protecting group was then removed from each by further reduction with sodium borohydride followed by acetic acid. As one stereocentre was removed with the protecting group, this resulted in the production of two diastereomers. Since this synthesis started with L-alanine, the two products were (2S, 3S)-2-amino-3-octadecanol (**154**) and (2S, 3R)-2-amino-3-octadecanol (**155**).

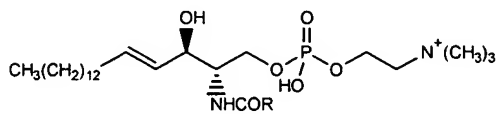
Biological Activity

While the spisulosines were quite simple compounds, as illustrated in Figures 1A- 1F, they exhibited a very unusual type of bioactivity. As discussed above, the spisulosines caused a distinct morphological change in L1210 leukemia cells, in addition to cytotoxicity. This bioactivity, which was recorded as the percentage of living cells in which altered morphology was observed, could be observed sometimes as early as 13 h after the start of the assay and reached a maximum at 50-60 h, after which it decreased. Generally 60 cells were observed to determine this number, except in assays in which less than this number of cells remained alive. The morphological effect was usually measured 30-35 h after the start of the assay and again about 24 h later, while the cytotoxicity was determined when the number of cells in the controls reached approximately 8000, usually in 3 days after the assay was begun. It should be noted that the pointed cells were live cells and that they were counted as such for the cytotoxicity reading. Also, assays in which 100% cytotoxicity was recorded may still have contained live cells (<0.5%) which may or may not have been pointed. All morphologically-changed cells were counted in the pointed cell percentage.

This change in morphology was always observed in fractions with fairly high cytotoxicity. Generally, no significant number of pointed cells were observed in assays with less than 70% growth inhibition. However, assays in which the cytotoxicity approached 100% often had lower percentages of cells with altered morphology than those with 90-98% growth inhibition. This suggested that the altered cells might be more easily killed. It is unknown whether the cytotoxicity and the morphology change resulted from the same mechanism of action. In one instance, pointed cells from an assay were recultured and found to revert to the normal state. This suggested that the effect was reversible after the compound had been

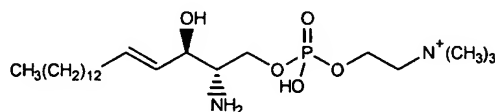
metabolized. Acetylation drastically reduces the bioactivity.

To determine if the change in morphology of L1210 cells was caused by sphingosine (**4**) or related compounds, several authentic compounds were obtained and assayed against L1210 cells. Both sphingosine and stearylamine (**131**) exhibited moderate cytotoxicity, but no morphological effect. Sphingomyelins are well-known derivatives of **4** in which a phosphorylcholine unit has been added to the primary alcohol and the amine is acylated by a fatty acid. A mixture of sphingomyelins isolated from bovine brain (Sigma), which consisted mainly of stearyl and nervonoyl sphingomyelins (**161**, **162**), showed minimal cytotoxicity and no pointed cells. The cytotoxicity of the phosphorylcholine derivative of **4** (**163**, Sigma) may be, at least, partially due to hydrolysis of **163** to **4**.



161 R = $-(\text{CH}_2)_{16}\text{CH}_3$

162 R = $-(\text{CH}_2)_{13}\text{CH}=\text{CH}(\text{CH}_2)_7\text{CH}_3$ (Z)



163

Cytotoxicity of Model Compounds

Compound	Concentration ($\mu\text{g/ml}$)	% Inhibition	% Pointed cells
128	5	100	0
	2.5	100	0
	1	75	0
	0.5	31	0
	0.25	13	0

	0.1	0	0
161+162	50	7	0
	25	0	0
	10	0	0
131	5	99	0
	2.5	96	0
	1	19	0
	0.5	0	0
	0.25	0	0
	0.1	0	0
163	50	88	0
	25	50	0
	10	38	0

Sphingosine and other long-chain amines, including stearylamine, are known to be cytotoxic. This bioactivity, as measured against Chinese hamster ovary (CHO) cells, has been shown to be maximal for 18-carbon homologues. All four stereoisomers of sphingosine were found to be almost equally active. Reduction of the double bond of **4** to produce dihydrosphingosine (**164**) did not affect the cytotoxicity. Addition of an N-methyl group to **164** also caused no significant change in the bioactivity, while acylation of the amine caused a large decrease in the cytotoxicity.

No cytotoxicity was reported for the related compounds (**134**, **135**, **140-142**) which

have been isolated from other marine sources, however, they may not have been tested in this type of assay. A mixture of **134** and **135** was active against *C. albicans* (8-mm zone of inhibition for 19 mg of a mixture of the two). Xestaminol A was reported to exhibit weak activity against several Gram-positive and Gram-negative bacteria and fungi. It also showed antihelminthic activity against *Nippostrongylus brasiliensis*. Both **140** and **142** showed some activity against reverse transcriptase.

The activity of fractions A-C, the acetyl derivative of fraction B and compounds **154** and **155** is summarized in the table. The assay results clearly confirmed the NMR analysis assigning **155**, not **154**, as the same as **125**. Also, acetylation drastically reduces the bioactivity.

Table IX. Bioactivity of Fractions A-C, AcB, and 154 and 155

Sample	Concentration ($\mu\text{g/ml}$)	Inhibition Inhibition ^c	Time	%Pointed Cells ^{a/b}	
				1st	2nd
Fraction A	2.5	100	35,59	ad	ad
	1.25	100		25	ad
	0.5	90		42	45
	0.25	85		45	55
	0.125	75		8	35
	0.05	19		0	0
Fraction B	2.5	100	35,59	0	7

Substitute Specification
Marked-up Copy

31

US 10/693,174
Filing Date: October 23, 2003

	1.25	93		3	21
	0.5	90		2	43
	0.25	80		7	37
	0.125	75		5	21
	0.05	7		0	0
Fraction C	2.5	90	55	0	
	1.25	88		0	
	0.5	63		0	
AcB	10	31	27	0	
	5	38		0	
	2	13		0	
	1	0		0	
	0.5	0		0	
	0.2	0		0	
154	5	100	27	0	
	2.5	100		0	
	1	63		0	
	0.5	0		0	
	0.25	0		0	
	0.1	0		0	
155	5	100	27	ad	
	2.5	100		22	
	1	100		64	

0.5	99	56
0.25	96	40
0.1	63	33

footnote

^aUnless otherwise indicated, the percentage of pointed cells was read twice. The number of hours after the start of the assay at which these measurements were made is indicated in the time column.

^b_{ad} = all dead.

^cThe percentage of growth inhibition which was recorded as the percentage of live cells in the treated wells compared to that in control wells.

Possible Mode of Action

The bioactivity of the spisulosines may be due to their similarity to sphingosine. In the nomenclature of sphingolipids, spisulosine 285 would be considered 1-deoxysphinganine. The spisulosines may compete with sphingosine for binding sites or be incorporated into sphingolipids such as sphingomyelins, ceramides or gangliosides. In either case, the spisulosines could disrupt the cellular functions controlled by these compounds. Sphingosine and its derivatives are involved in the regulation of cell growth and differentiation. Sphingosine is a potent inhibitor of protein kinase C, competing with diacylglycerol for the binding site, which may explain its cytotoxicity. Structure-activity studies have shown that this inhibition requires a positively charged amine and thus *N*-acyl derivatives were inactive. If the spisulosines act by

competing with sphingosine, this would explain the relative lack of activity of the acetylated compounds (AcB). There is growing evidence that sphingosine may act as a second messenger by regulating protein kinase C activity. It has also been shown to inhibit the differentiation of HL-60 cells treated with phorbol 12-myristate-13-acetate, a known protein kinase C activator. The spisulosines should be tested for inhibition of protein kinase C. It is unknown whether inhibition of this enzyme could cause the morphological effects observed for the spisulosines, but protein kinase C is involved in the control of cell growth and differentiation.

Experimental

NMR spectra were obtained on General Electric GN 500 and QE 300 and Varian U400 spectrometers. Samples for NMR analysis were dissolved in CDCl₃ or CD₃OD. Chemical shifts (δ) are reported in ppm downfield of tetramethylsilane (TMS) and referenced to the residual solvent peak or TMS. Low and high resolution FABMS spectra were recorded on either a VG ZAB-SE or a VG 70-SE4F spectrometer, using a 3:1 mixture of dithiothreitol -dithioerythritol (magic bullet) as the matrix. FABMS/MS spectra were recorded on a VG 70-SE4F with the same matrix, using helium as the collision gas. CI mass spectra were recorded on a VG VSE spectrometer, operating in the alternating CI/EI mode with methane as the reagent gas. IR spectra were obtained on an IBM IR/32 FTIR spectrometer. Optical rotations were measured on a JASCO DIP-370 digital polarimeter.

Chromatography

HPLC was carried out using an Alltech Econosphere cyano column (4.6 x 250 mm, 5 gm particle size). The HPLC system used consisted of a Beckman Model 114M pump, a Rheodyne 71 injector and either an Isco V⁴ or Beckman 165 variable wavelength detector or a Waters 990 photodiode array detector.

Analytical thin layer chromatography (TLC) was performed on a pre-coated silica gel (Merck 60 F-254) and cyano bonded-phase (EM Science CN F-254 F_{254S} HPTLC) plates. Spots were visualized by UV (254 nm), ninhydrin (5% in ethanol), phosphomolybdic acid (5% in ethanol) and/or iodine. Silica column chromatography was carried out on either 50-200 mm or 40-63 mm silica gel (Merck). Other column chromatography used Chromatorex ODS (Fuji-Division 100-200 mesh) and Sephadex LH-20 (Pharmacia). High speed countercurrent chromatography (HSCCC) was performed on an Ito multi-layer coil separator-extractor (P.C., Inc.) with a #10 coil and a Milton-Roy mini-Pump. Solid phase extraction (SPE) was carried out on normal phase (silica, Alltech Maxi-Clean), reversed-phase (C-18, Waters Sep-Pak), and bonded-phase (CN, Fisher PrepSep) columns.

Biological Assays

Cytotoxicity assays against L1210 murine lymphocytic leukemia cells were performed by dissolving the samples in methanol and/or hexane were applied to the dry assay wells and the solvent was allowed to evaporate. Cells (1000) were added in minimum essential medium (MEM, 1 ml) and incubated 37 °C. Inhibition of growth was recorded as the estimated percentage of living cells in sample wells versus that in control wells. This was measured when

the control wells reached 8000 cells, generally three days after the start of the assay.

Morphologically-changed cells (Figures 1A-1F) were counted as living cells when determining the ~~per-cent~~ percent inhibition of growth. Morphological changes were assessed throughout the assay period. The percentage of pointed cells was determined by counting the number of altered cells in approximately 60 living cells. This percentage varied with the length of time the assay had been running. It generally reached its maximum about 50 hours after the start of the assay, but pointed cells could be observed as early as 13 hours after the start of the assay and could usually still be seen when the percent growth inhibition was measured. The percentages of pointed cells were often counted both after about 35 and after 55 hours. The time that this measurement was made is indicated with the data.

Antimicrobial assays were performed using the filter disk diffusion method. Paper disks (6.35 or 12.7 mm, Schleicher & Schuell) were impregnated with samples (50-500 µg) in solution and allowed to dry. These disks were then placed on agar seeded with either *Bacillus subtilis*, *Penicillium melinii* (formerly *P. atrovenetum*), *Micrococcus luteus* (formerly *Sarcina lutea*), *Escherichia coli* or *Saccharomyces cerevisiae*. These plates were incubated for 12-24 h (32-35 °C, except *P. melinii*, 25-27 °C).

Extraction of *Spisula polynyma* for Initial Biological Testing

One clam (*Spisula polynyma*) was thawed and the shell removed (35.32 g, wet wt). This was placed in a blender with 350 ml of 3:1 methanol/toluene and homogenized. The yellow-

brown extract was filtered and added to a 1M sodium chloride solution (100 ml). The upper toluene layer was removed and the aqueous layer extracted with toluene (75 ml). The two toluene layers were combined and the solvent was removed to give a brown oily residue (333.9 mg). The aqueous layer was further extracted with dichloromethane (2.times.75 ml), which gave a yellow-brown residue (18.6 mg) after removal of the solvent. The aqueous layer was then extracted with ethyl acetate (75 ml). The lower phase was the organic layer due to the presence of some dichloromethane which had remained in the aqueous phase after the last step. The upper layer was further extracted with the ethyl acetate (245 ml), the upper organic layer back-extracted with water (100 ml), and the two ethyl acetate extracts were combined to give a yellow residue (36.8 mg) after removal of the solvent. The combined aqueous layers were concentrated by one-half and extracted twice with 1-butanol (150 ml, 75 ml). The combined butanol layers were back-extracted with water (75 ml), resulting in a yellow residue (132.8 mg) after removal of the butanol. The combined aqueous layers were concentrated to give an oily light yellow residue (946.1 mg). Each extract was triturated with dichloromethane and methanol to remove salts to give the toluene (302.2 mg), dichloromethane (18.6 mg), ethyl acetate (36.7 mg), butanol (120.9 mg) and aqueous (590.4 mg) extracts which were assayed.

Fractions A, B, and C

Thirty-five clams were thawed and the shells removed to give a sample of *Spisula polynyma* (1.9 kg) which was soaked in methanol/toluene (3:1, 2 x 1.5 l). The solids were then ground in the same solvent (6 x 1.5 l) and the resulting extracts filtered. A 1 M solution of sodium chloride (3 l) was added to this crude extract (12 l) and the resulting upper toluene layer

removed. The aqueous layer was further extracted with toluene (2 x 2.5 l), followed by dichloromethane (4 x 2.5 l) as shown in Figure 1.

After removal of the solvent, the toluene extract (21.55 g) was partitioned between methanol and hexane (1.5 l each). The methanol layer was further extracted with hexane (4 x 1 l). The combined hexane layers were concentrated to about 1.8 l and both extracts chilled (-10 °C). The two layers which resulted in each case were separated. The combined hexane layers were then extracted with methanol (0.5 l). This process resulted in a hexane and three methanol extracts of which the first methanol extract (6.8 g) contained the most bioactivity.

This bioactive methanol fraction was separated by flash silica chromatography employing a chloroform/methanol step gradient (100:0, 99:1, 95:5, 90:10, 85:15, 80:20, 70:30, 50:50, 0:100) to give 12 fractions. While the third, fourth, seventh and eighth fractions possessed some cytotoxicity, they showed no pointed-cell forming activity. This activity was found in the last two fractions along with most of the cytotoxicity.

These two fractions were combined (370 mg) and further purified by another flash silica column, using chloroform/1-butanol/acetic acid/water (8:12:1:1). To remove the acetic acid, each of the 12 fractions thus obtained was neutralized by (a) adding chloroform (one-quarter volume), (b) washing with 5% sodium bicarbonate until the pH of the aqueous layer was above 7 (2-3 x half volume), and then (c) washing the organic layer with water (half volume). The third, and fourth and fifth fractions possessed all of the pointed cell-forming activity and essentially all of the cytotoxicity. Each of these fractions was separately purified by HPLC on a

cyano column with 3:1 methanol/0.01 M ammonium formate (1 ml/min). Six fractions, of which the most bioactive was the fifth, were collected from each silica fraction. The ammonium formate was removed from each fraction by adding water (2-8 ml), applying the sample to an SPE column (C-18), washing with water (5-10 ml) and then eluting with methanol (5 ml). This resulted in fractions A (0.4 mg, 2×10^{-5} % yield), B (1.3 mg, 7×10^{-5} % yield) and C (0.2 mg, 1×10^{-5} % yield), from the third, fourth and fifth silica fractions, respectively, which all eluted at t_r 7.9 min.

Fraction A

White solid; silica TLC (3:12:2:2 CHCl₃/1-BuOH/AcOH/H₂O) R_f 0.47 (ninhydrin-positive, pink); IR (NaCl) 2922, 2853, 1734, 1593, 1462, 1377, 1061 cm⁻¹; ¹H NMR (CDCl₃) δ 5.38, 5.15, 3.82, 3.67, 3.44, 3.24, 2.31, 2.03, 1.67, 1.60, 1.55, 1.25, 1.10, 0.86; FABMS m/z 606, 604, 592, 590, 466, 452, 438, 314, 300, 286, 268; CIMS m/z 354, 340, 338, 328, 326, 324, 314, 312, 310, 300, 298, 296, 286, 284, 268, 266, 149, 139, 137, 1, 123, 111, 109, 97, 95, 85, 83, 71, 69, 59, 57, 55. Anal. Calcd. For C₁₈H₄₀NO: 286.3110 (M+H). Found: 286.3115 (HRFABMS).

Fraction B

White solid; silica TLC (3:12:2:2 CHCl₃/1-BuOH/AcOH/H₂O) R_f 0.47 (ninhydrin-positive, pink), 0.44 (ninhydrin-positive, red); IR (NaCl) 3273, 2953, 2918, 2851, 1639, 1591, 1510, 1466, 1379, 1344, 1059, 970 cm⁻¹; ¹H NMR (CDCl₃) δ 5.98, 5.78, 5.55, 5.44, 5.32, 4.43, 3.78, 3.65, 3.24, 2.15, 2.08, 2.00, 1.95, 1.70, 1.44, 1.25, 1.19, 0.87; FABMS m/z 618.2940, 616,

606.2955, 604.2831, 592, 590, 480, 466, 464.2888, 452.2885, 438, 314.3439, 314.3075, 300.3273, 300.2914, 286, 268; CIMS m/z 354, 352, 342, 340, 338, 328, 326, 324, 314, 312, 310, 300, 298, 296, 286, 284, 282, 280, 268, 266, 219, 193, 179, 165, 149, 137, 123, 111, 109, 97, 95, 85, 83, 71, 69, 59, 57, 55.

Fraction C

White solid; silica TLC (3:12:2:2 CHCl₃/1-BuOH/AcOH/H₂O) R_f 0.47 (ninhydrin- positive, pink), 0.44 (ninhydrin-positive, red), 0.34 (ninhydrin- positive, purple); IR (NaCl) 2924, 2853, 1593, 1456, 1352, 1063, 972 cm⁻¹; FABMS m/z 620, 618, 616, 606, 604, 602, 466, 464, 452, 438, 314, 300, 298.2741, 296, 286, 280, 268; CIMS m/z 354, 352, 340, 338, 336, 328, 326, 324, 322, 314, 312, 310, 308, 300, 298, 296, 294, 292, 286, 284, 282, 280, 278, 268, 179, 165, 149, 137, 135, 1, 123, 121, 111, 109, 97, 95, 85, 83, 81, 71, 69, 60, 59, 57, 55.

Initial Partitioning

Twenty-two *S. polynyma* claims were thawed and the shells removed to give 1.3 kg of the organism (wet wt). This was placed in Waring blender with 3:1 methanol/toluene (1.5 l) and ground into a thick slurry which was filtered through a layer of celite. The solid residue was further extracted (4x1.5 l) and filtered in a similar manner. The remaining solids were then placed in 5:1 methanol/toluene (750 ml) and allowed to soak for 36 h, before filtering. To the combined filtrates (7.8 l) was added 1 M sodium chloride (2 l). After removal of the upper toluene layer, the aqueous phase was extracted with toluene (2 x 1.5 l) and dichloromethane (3 x

1.5 l). The remaining aqueous phase was concentrated by one-half and extracted with ethyl acetate (2 x 1 l). The resulting aqueous layer was diluted with water (2 l) and extracted twice with 1-butanol (1.5 l, 1 l). Removal of the solvents and trituration with dichloromethane and methanol resulted in the toluene (14.1 g), dichloromethane (0.75g), ethyl acetate (1.3 g), 1-butanol (0.2 g) and aqueous (1.9 g) extracts which were assayed.

The toluene extract was partitioned between hexane and methanol (750 ml each). The resulting methanol layer was further extracted with hexane (2 x 750 ml, 2 x 500 ml). The hexane layers were combined and concentrated to about 3 l and then both extracts were chilled (-10 °C) which caused each to separate into two layers. The combined methanol layers were concentrated in vacuo to give a brown residue (methanol extract 1, 536 g). The hexane layers were further concentrated to about 1 l and back- extracted with methanol (500 ml). The solvent was removed from each of these to give the methanol extract 2 (4.26 g) and the hexane extract (4.52 g).

Fraction D

A portion of the first methanol extract (594 mg) was separated by HSCCC, using hexane/ethyl acetate/methanol/water (4:7:4:3, MP = UP) at 4 ml/min. This gave 12 fractions of which the third, fourth and fifth contained most of the bioactivity. These three fractions were combined (158 mg) and chromatographed on Sephadex LH-20, eluting with methanol. This resulted in eight fractions of which the fourth (8.4 mg) possessed the majority of the biological activity. This bioactive fraction was further purified by HPLC on a cyano column with 3:1 methanol/0.01 M ammonium formate (0.5 ml/min). Eight fractions were collected and the

ammonium formate was removed from each by adding water (2-8 ml), applying the sample to an SPE column (C- 18), washing with water (5-10 ml) and then eluting with methanol (5 ml). The seventh fraction (t_r 15.8 min, white amorphous solid, 0.3 mg, 2×10^{-4} % yield) proved to contain the bioactive compounds and is referred to here as fraction D. Silica TLC (1-BuOH/AcOH/H₂O, 4:1:5, upper layer) showed four spots by phosphomolybdic acid visualization: R_f 0.53 (major), 0.35 (major), 0.31 (minor), and 0.19 (minor). The inactive sixth fraction showed all the same spots except R_f 0.53. The FABMS spectrum of fraction D showed intense peaks at m/z 286.3019, 300.3270 and 268.3019, and weaker peaks at m/z 314, 438, 452, 464, 590, 592, 669, 797, 809 and 825. The last three ions listed were also observed in most of the other HPLC fractions and appeared to correspond to the TLC spot at R_f 0.35.

Anal. Calcd. ~~For~~for C₁₈H₄₀NO: 286.3110 (M+H). Found: 286.3109 (HRFABMS).

Fraction E

A second portion of the first methanol extract described above (633 mg) was subjected to HSCCC. The solvent system employed was hexane/methanol/water (5:4:1, UP = MP, 5 ml/min), which gave poor stationary phase retention. This resulted in 10 fractions with the bioactivity spread throughout most of them. The first three fractions (310 mg) were combined and further purified by HSCCC using hexane/ethyl acetate/methanol/water (4:7:4:3, LP = MP, 2 ml/min) to give 12 fractions. The second to fifth fractions (85 mg), containing the majority of the bioactivity, were chromatographed on a C-18 flash column, eluting with a methanol/water/chloroform step gradient (90:10:0, 95:5:0, 100:0:0, 95:0:5, 90:0:10, 50:0:50).

This gave 10 fractions which were all bioactive.

The fourth to sixth fractions from the first HSCCC run were combined with a side fraction from the Sephadex LH-20 column discussed under fraction D (270 mg). This material was subjected to HSCCC, using the same conditions as the second run just described except that the flow rate was 3 ml/min. This resulted in nine fractions of which the second and third contained most of the cytotoxicity and cell-altering activity. These two fractions were combined (42 mg) and separated on a flash C-18 column, using a methanol/water step gradient (80:20, 90:10, 95:5, 100:0). This resulted in 12 fractions of which the eighth to eleventh showed morphology-altering activity and cytotoxicity. All but the first and fifth fractions from the first C-18 column were combined with the eighth to eleventh fractions from the second (50.4 mg) and separated by preparative silica TLC with chloroform/ 1-butanol/acetic acid/water (3:12:2:2). The plate was divided into eight fractions, which were scraped off and eluted with methanol. The residue from each fraction after removal of the solvent was triturated with dichloromethane and filtered. The fraction second from the top of the plate (R_f 0.80-0.42) contained the bioactive material and is referred to as fraction E (5.7 mg). Analytical silica TLC of fraction E, eluting with the same solvent system, showed a single spot by ninhydrin visualization (R_f 0.44), but phosphomolybdic acid spray reagent showed other material which streaked throughout the middle third of the plate. The FABMS spectrum of fraction B showed m/z 286 as the major peak, with lesser peaks at m/z 268, 300, 438, 452, and 592.

Fraction F

A third portion of the first methanol extract (468 mg) was separated by flash silica chromatography, using the solvent system chloroform/1-butanol/acetic acid/water (8:12:1:1). To remove the acetic acid, each of the 10 fractions thus obtained was neutralized by (a) adding water (half the volume of the fraction) and separating the two phases, (b) extracting the aqueous layer with chloroform (half volume x 2), (c) washing the combined organic layers with 5% sodium bicarbonate until the pH of the aqueous layer was above 7 (2 to 3 x half volume), and then (d) washing the organic layer with water (half volume). The third fraction (24 mg), which possessed the majority of the bioactivity, was chromatographed on Sephadex LH-20, eluting with methanol, to give eight fractions. The sixth fraction (2.3 mg) was separated by repeated HPLC, using the same conditions as for the separation of fraction A-C. The ammonium formate was removed as for fraction A-C. The fraction eluting at t_r 8.1 min was the most biologically active and is referred to as a fraction F. It was so small that an accurate weight could not be obtained, but probably was 100-200 μg (approximately 1 to 2 x 10^{-4} % yield). The fractions eluting later than this one also showed both cytotoxic and pointed cell-forming activity, although less potent. This suggested that either the bioactive compound(s) did not elute as a well-defined peak or that different homologues eluted at different times, but were not well separated. Silica TLC (3:12:2:2 CHCl_3 /1-BuOH/AcOH/ H_2O) showed one ninhydrin-positive spot at R_f 0.44. The later eluting fractions also showed this same spot, but less intense. The FABMS spectrum of fraction F shows (in decreasing order of intensity) m/z 286, 268, 300, 314, 344, 438, 452, 592, 669.

Dissection

A live clam was placed in a container with about 10 ml of diethyl ether and chilled (4 °C) for 20 h. It was dissected into nine organs: foot, digestive system (including the stomach, intestines and crystalline style sac), gonads, siphon, gills, heart, mantle, adductor muscles, and the remainder of the visceral mass. Each organ was first soaked in methanol/toluene (3:1, 10 ml/g sample) and then homogenized in a Virtis blender. The extracts were filtered and the solvent was removed. The residue was triturated with dichloromethane and methanol to give 155 mg (foot), 60 mg (digestive system), 147 mg (gonads), 101 mg (siphon) 65 mg (gills), 2.5 mg (heart), 168 mg (mantle), 101 mg (adductor muscles) and 252 mg (visceral mass).

In a separate experiment, one foot that had been cooked was extracted in an analogous fashion (189 mg). A larger sample of cooked clams (483 g) was more extensively extracted by first soaking in 3:1 methanol/toluene (3 x 500 ml) and then homogenizing the sample in the same solvents (5 x 500 ml). A small sample of the combined extracts was evaporated and redissolved in methanol for assaying.

General Procedures

Optical rotations were measured on a Jasco DIP-370 digital polarimeter, with a 3.5 X 50 mm 1 ml cell. Melting points were taken on a Thomas Hoover capillary melting point apparatus. ¹H and ¹³C NMR were recorded on a Varian Unity- 400 or Unity -500 spectrophotometer. Chemical shifts are reported in ppm relative to the solvent (7.26, CDCl₃ and 3.30, CD₃OD). High resolution (HRFAB) and fast atom bombardment (FAB) mass spectra were recorded on a VG ZAB-SE or a 70 SE4F mass spectrometer. TLC was done on Merck Silica Gel

60 Thin-Layer Plates. Chromatographic separations were done by flash chromatography using 230-400 mesh Merck silica gel. All moisture sensitive reactions were run in oven-dried glassware under an atmosphere of N₂. Solvents were distilled prior to use: THF from benzophenone ketyl, CH₂Cl₂ from CaH₂ other solvents used were reagent grade.

(S)-2-(N,N-Dibenzylamino)propionic acid methyl ester (30):

To a 300 ml round bottom was added **20** (10.0 g, 71.6 mmol), benzyl bromide (25.73 g, 150.4 mmol), K₂CO₃ (9.90 g, 71.6 mmol) and CH₃CN (172 ml). The mixture was stirred at 60 °C until the reaction was complete by TLC. The reaction was cooled to room temperature and the solid was separated by filtration. The filtrate was concentrated in vacuo to give an oil which was purified by flash chromatography on silica gel (9:1 hexane/EtOAc) to give a colorless oil:

$[\alpha]_D^{25} = +113.6$ (c 1.2, CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ 1.35 (d, 3H, J = 7.1 Hz), 3.53 (q, 1H, J = 7.0 Hz), 3.65 (d, 2H, J = 1.38 Hz), 3.75 (s, 3H), 3.85 (d, 2H, J = 13.8 Hz), 7.22-7.42 (m, 10H); ¹³C NMR (100 MHz) δ 14.9, 51.1, 54.3, 56.0, 2.8, 4.1, 4.5, 139.1, 175.1; FABMS *m/z* 284.1 (M+H), 282.1 (M-H), 224.2 (M-COOCH₃); HRFABMS calcd for C₁₈H₂₂NO₂M_r 284.165.1 (M+H), found M_r 284.1650.

(S)-2-(N,N-Dibenzylamino)-1-propanol (40):

To a suspension of LiAlH₄ (550 mg, 14.5 mmol) in THF (20 ml) a solution of **30** (910 mg, 3.21 mmol) in THF (2 ml) was added dropwise. The solution was stirred for 15 minutes and then heated to 65 °C for 3 hours. The reaction was cooled to 0 °C and quenched with 0.1 N HCl.

The reaction was filtered through Celite and the Celite washed with THF (2x15 ml) and the solvent removed in vacuo. Flash chromatography on silica gel (4:1 hexane/EtOAc, $R_f = 0.30$) gave 750 mg (92% yield) of a colorless solid: mp 40-41 °C (from hexane) Literature mp 40-41 °C (from hexane) See, Stanfield et al., *J Org. Chem.* 1981, 49, 4799-4800; $[\alpha]_D^{25} = +86.6$ (c 1, CHCl₃) Literature $[\alpha]_D^{25} = +88.2$ (c 1, CHCl₃); ¹H NMR (500 MHz CDCl₃) δ 0.98 (m, 3H), 2.98 (m, 1H), 3.13 (m, 1H), 3.35 (m, 3H), 3.45 (m, 1H), 3.81 (m, 2H), 7.19-7.41 (m, 10H); ¹³C NMR (1 MHz) δ 8.6, 52.9, 54.1, 62.7, 3.2, 4.5, 5.0, 5.3; FABMS m/z 256.2 (M+H), 224.2 (M-CH₂OH); HRFABMS calcd for C₁₇H₂₂NO M_r 256.1701 (M+H), found M_r 256.1702.

(S)-2-(N,N-Dibenzylamino)propionaldehyde (50):

Dry DMSO (0.53 ml, 7.43 mmol) was added to a stirred solution of oxalyl chloride (0.31 ml, 3.6 mmol) in CH₂Cl₂ (7.5 ml) at -78 °C. The solution was allowed to stir 15 minutes followed by the addition of **40** (740 mg, 2.90 mmol) in CH₂Cl₂ (7.5 ml). After 30 minutes, Et₃N (1.0 ml, 7.2 mmol) was added and allowed to warm to room temperature. The solution was extracted with saturated. NaHCO₃ (20 ml) and the aqueous layer was extracted with CH₂Cl₂ (2 x 15 ml). The organic layer was washed with saturated. NaCl solution, dried with MgSO₄ and concentrated in vacuo at room temperature to give 720 mg (98% yield) of a yellow oil which became a solid when cooled to -20 °C. The aldehyde was used without further purification: mp 52-54 °C, Literature mp 55.5 °C See, Dix et al., *Arch Pharm (Weinheim)* 1995, 328, 203-205.; $[\alpha]_D^{26} = -36.0$ (c 1, CHCl₃) Literature $[\alpha]_D^{20} = -35.1$ (c 1, EtOAc); ¹H NMR (400 MHz, CDCl₃) δ 1.19 (d, 2H, J= 7.0 Hz), 3.34 (q, 1H, J= 7.0 Hz), 3.58 (dd, 2H, J= 13.7 Hz), 3.74 (d, 2H, J= 13.7 Hz), 7.26 (m, 2H), 7.33 (m, H), 7.42 (m, 4H), 9.74 (s, 1H); ¹³C NMR (100 MHz) δ 6.7,

54.9, 62.8, 3.3, 4.4, 4.8, 139.1, 204.6; FABMS m/z 408.2 (M+MB), 254.2 (M+H), 22.2 (M-CHO); HRFABMS calcd for $C_{17}H_{20}NO$ M_r 254.1545 (M+H), found M_r 254.1545.

(2S,3R)-2-(N,N-Dibenzylamino)-3-octadecanol (60):

Mg ribbon (237 mg, 9.75 mmol), dibromoethane (16 μ L, 0.189 mmol) in THF (160 μ L) were added to a two neck flask fitted with a reflux condenser. A 1/2 ml of a 1-bromopentadecane solution (970 mg, 3.33 mmol, 3.25 ml THF) was added. After the reaction had started the remainder was added dropwise. To the grayish solution, **50** (105 mg, 0.413 mmol) in THF (0.5 ml) was added dropwise. The reaction was allowed to stir overnight followed by the addition of H_2O (5 ml) and 0.1 N HCl until the solution became clear. The mixture was extracted with EtOAc (3 x 10 ml). The organic layer was washed with 5% $NaHCO_3$ then saturated. NaCl solutions and dried with $MgSO_4$. The solvent was removed in vacuo to give an oil-solid mixture (750 mg). The crude material was purified by flash chromatography on silica (8:1 hexane/EtOAc, R_f = 0.34) to give 120 mg of a solid. This solid was further purified by HPLC on silica (93:7 hexane/EtOAc) to give a colorless waxy solid (94.3 mg, 49% yield): $[\alpha]_D^{25} = +16.3$ (c 1, $CHCl_3$); 1H NMR (500 MHz, $CDCl_3$) δ 0.88 (t, 3H, J = 7.0 Hz), 1.10 (d, 3H, J = 6.7 Hz), 1.16-1.41 (bm, 26H), 1.56 (m, 1H), 1.69 (m, 1H), 1.79 (m, 1H), 2.72 (quin, 1H, J = 6.7 Hz), 3.47 (d, 2H, J = 13.8 Hz), 3.60 (m, 1H), 3.76 (d, 2H, J = 13.8 Hz), 7.22 (m, 2H), 7.30 (m, 4H), 7.34 (m, 4H); ^{13}C NMR (1 MHz) δ 8.67, 14.11, 22.68, 25.90, 29.35, 29.61, 29.64, 29.68, 29.69, 31.91, 34.27, 54.79, 57.26, 73.65, 78.89, 84.25, 84.77, 140.17; FABMS m/z 465 (M+H), 448 (M- H_2O), 464 (M-H), 388 (M-Ph), 224 (M- $C_{16}H_{33}O$); HRFABMS calcd for $C_{32}H_{52}NO$ M_r 466.4049 (M+H), found M_r 466.4037.

The assignment of the 2S,3R configuration is based on comparison of the chemical shifts of the benzyl protons in **60** to literature values for the *syn* and *anti* diastereomers of 2-(N,N-dibenzylamino)-3-pentanol. The *anti* isomer has a chemical shift difference of 0.29 ppm. and the *syn* is 0.52 ppm. Comparison of other *syn-anti* pairs show the range for the *syn* isomer to be 0.44 to 0.54 ppm and the *anti* 0.05 to 0.29 ppm. The value for **60** is 0.29 ppm.

(2S,3R)-2-Amino-3-octadecanol (1):

To a 15 ml round bottom was added **60** (88.2 mg, 0.189 mmol) in MeOH (2 ml) and 20% Pd (OH)₂-C (11 mg). The mixture was stirred under 1 atmosphere of hydrogen overnight. The catalyst was removed by filtration through a 25 mm syringe filter (0.2 μ m nylon membrane) and the filter was washed with 4 ml of MeOH. The solvent was then removed in vacuo to give 51.50 mg of a white solid. The product was purified by chromatography over a 6 ml LC-Si SPE tube (90:10 CH₂Cl₂/MeOH followed by 100% MeOH) to give 49.47 mg (92% yield) of a white solid: mp 66-67 °C; [α]_D²⁶ = +24.9 (c 1, CHCl₃); ¹H NMR (500 MHz, CD₃OD) δ 0.89 (t, 3H, J = 7.0 Hz), 1.05 (d, 3H, J = 6.6 Hz), 1.20- 1.56 (bm, 31H), 2.81 (qd, 1H, J₁=6.6 Hz, J₂ = 3.8 Hz), 3.42 (dt, 1H, J₁ =8.8 Hz, J₂ = 3.8 Hz); ¹³C NMR (1 Mhz) δ 14.60, 16.82, 23.90, 27.40, 30.65, 30.90, 30.95, 30.96, 33.23, 34.13, 52.33, 76.16,; FABMS *m/z* 286.3 (M+H), 268.3 (M-OH), HRFABMS calcd for C₁₈H₄₀NO M_r 286.3110 (M+H), found M_r 286.3109.

A mixture of diastereomers of 3-hydroxy-2-(1-methyl-2-2-hydroxy-heptadecyl)-isoindolin-1-one (**152**, 22 mg) were separated by cyano HPLC with hexane/2-propanol (98:2, 1

ml/min) to give four compounds (**152a-152d**). The purity of each peak was determined by reinjection on HPLC. Anal Calcd. For $C_{26}H_{44}NO_3$: 418.3321 (M+ H). Found: 418.3321 HRFABMS).

152a: 4.2 mg; t_r 13.3 min; 1H NMR ($CDCl_3$) δ 7.77 (1 H, d, 7.3), 7.58 (2H, m), 7.50 (1H, m), 5.91 (2H, s), 4.51 (1H, m), 3.78 (1H, m), 1.58 (2H, m), 1.40 (3H, d, 7.1), 1.24 (26H, m), 0.87 (3H, t, 6.5); FABMS m/z 418, 400; relative ratio of diastereomers 17:1:0:0 (**152a:152b:152c:152d**).

152b: 13.7 mg; t_r 13.9 min; 1H NMR ($CDCl_3$) δ 7.70 (1 H, d, 7.3), 7.54 (2H, m), 7.47 (1H, m), 5.88 (2H, s), 4.37 (1H, m), 3.85 (1H, m), 1.52 (2H, m), 1.27 (3H, d, 7.0), 1.25 (26H, m), 0.87 (3H, t, 6.5); FABMS m/z 418, 400; relative ratio of diastereomers 1:6.8:0:0 (**152a:152b:152c:152d**).

152c: 1.4 mg; t_r 20.0 min; 1H NMR ($CDCl_3$) δ 7.78 (1H, d, 7.3), 7.59 (2H, m), 7.51 (1H, m), 5.93 (2H, s), 4.12 (1H, m), 3.99 (1H, m), 1.58 (2H, m), 1.37 (3H, d, 7.0), 1.25 (26H, m), 0.87 (3H, t, 6.5); FABMS m/z 418, 400; relative ratio of diastereomers 0:2.5:45:1 (**152a:152b:152c:152d**).

152d: 1.5 mg; t_r 21.7 min; 1H NMR ($CDCl_3$) δ 7.77 (1H, d, 7.3), 7.59 (2H, m), 7.51 (1H, m), 5.86 (2H, s), 4.12 (1H, m), 3.90 (1H, m), 1.58 (2H, m), 1.45 (3H, d, 6.6), 1.24 (26H, m), 0.87 (3H, t, 6.5); FABMS m/z 418, 400; relative ratio of diastereomers 0:1:2:21 (**152a:152b:152c:152d**).

Each diastereomer was separately deprotected by the method of Osby et al. Each isomer was dissolved in 2-propanol/water (6:1, 0.1 M for **152a** and **152b**, 0.7 M for **152c** and **152d**). Sodium borohydride (5-10 equivalents) was added to each solution, which was then stirred at 25 °C for 24 h. Each solution was then adjusted to pH 4.5 with acetic acid and stirred at 80 °C for an additional 24 h. Ammonium formate was added to bring the pH of each solution to above 7 and then the solvent was removed from each by a stream of nitrogen. The residue from each was applied to a silica SPE column, which was first washed with hexane:2-propanol (9:1) and then the product eluted with 2-propanol. ¹H NMR indicated that **152a** and **152d** produced **154** (1.15 mg, 40%, and 0.48 mg, 47% respectively), while **152b** and **152c** produced **155** (3.35 mg, 42%, and 0.38 mg, 40%, respectively).

154: White solid; silica TLC (3:12:2:2 CHCl₃/1-BuOH/AcOH/H₂O) R_f 0.48 (ninhydrin-positive, pink); IR (NaCl) 2919, 2851, 1563, 1466, 1406, 758 cm⁻¹; FABMS *m/z* 438, 286, 268, 85, 70, 69, 57, 55, 44. Anal. Calcd. For C₁₈H₄₀NO: 286.3110 (M+H). Found 286.3115 (HRFABMS).

155: White solid; silica TLC (3:12:2:2 CHCl₃/1-BuOH/AcOH/H₂O) R_f 0.50 (ninhydrin-positive, pink); IR (NaCl) 3281, 2917, 2849, 1568, 1520, 1470, 1412 cm⁻¹; FABMS *m/z* 438, 286, 268, 85, 70, 69, 57, 55, 44, Anal. Calcd. For C₁₈H₄₀NO: 286.3110 (M+H). Found: 286.3109 (HRFABMS).

Acetylation

A portion of fraction B (560 μ g) dissolved in acetic anhydride (200 μ L) and pyridine (400 μ L) and was stirred at 25 °C for 4.5 h, at which time no starting material could be observed by TLC. The solvent was removed by a stream of nitrogen to give AcB: off-white solid; silica TLC R_f 0.86 (3:12:2:2 CHCl_3 /1-BuOH/AcOH/ H_2O , phosphomolybdic acid), 0.65 (9:1 CHCl_3 /MeOH, phosphomolybdic acid); IR (NaCl) 2922, 2853, 1741, 1651, 1547, 1460 1371, 1234, 1022, 970 cm^{-1} ; FABMS m/z 370, 310, 268; CIMS m/z 426, 424, 412, 410, 398, 384, 370, 368, 364, 338, 324, 310, 165, 149, 139, 1, 121, 111, 97, 86, 61, 57, 55. Anal. Calcd. For $\text{C}_{22}\text{H}_{44}\text{NO}_3$: 370.3321(M+H). Found: 370.3326 (HRFABMS).

Triacetylsphingosine (133)

In a procedure similar to Grode and Cardellina D-*erythro*-sphingosine (4, 2 mg, 6.7 μ mol, Sigma) in acetic anhydride (1 ml) and pyridine (2 ml) was stirred at 25 °C for 4.5 h, at which time no starting material could be observed by TLC. The solvent was removed by a stream of nitrogen to give **133**: white solid; silica TLC R_f 0.86 (3:12:2:2 CHCl_3 /1-BuOH/AcOH/ H_2O , phosphomolybdic acid), 0.65 (9:1 CHCl_3 /MeOH, phosphomolybdic acid); FABMS m/z 580, 426, 366, 306, 264; CIMS m/z 468, 454, 426, 424, 394, 366, 364, 306, 264, 144, 85, 84, 83, 61.

(2S, 3S)-2-Acetamido-3-acetoxyoctadecane (156)

(2S, 3S)-2-amino-3-octadecanol (**154**, 150 μg , 0.5 μmol) in acetic anhydride (50 μl) and pyridine (100 μl) was stirred at 25 °C for 5 h, at which time no starting material could be observed by TLC. The solvent was removed by a stream of nitrogen to give **156**: white solid; silica TLC R_f 0.86 (3:12:2:2 CHCl_3 /1-BuOH/AcOH/ H_2O , phosphomolybdic acid); IR (NaCl) 3286, 2924, 2853, 1740, 1653, 1541, 1456, 1371, 1238 cm^{-1} ; FABMS m/z 522, 370, 328, 310, 286, 268. Anal. Calcd. For $\text{C}_{22}\text{H}_{44}\text{NO}_3$: 370.3321 (M+H). Found: 370.3326 (HRFABMS).

(2S, 3R)-2-Acetamido-3-acetoxyoctadecane (157)

(2S, 3R)-2-amino-3-octadecanol (**155**, 750 μg , 2.6 μmol) in acetic anhydride (200 μL) and pyridine (400 μL) was stirred at 25 °C for 5h, at which time no starting material could be observed by TLC. The solvent was removed by a stream of nitrogen to give **157**: white solid; silica TLC R_f 0.86 (3:12:2:2 CHCl_3 /1-BuOH/AcOH/ H_2O , phosphomolybdic acid); IR (NaCl) 3289, 2917, 2849, 1728, 1637, 1545, 1464, 1369, 1240 cm^{-1} ; FABMS m/z 522, 370, 328, 310, 286, 268. Anal. Calcd. For $\text{C}_{22}\text{H}_{44}\text{NO}_3$: 370.3321 (M+H). Found: 370.3319 (HRFABMS).

Spisulosine 285 Acetonide (146)

A portion of fraction A (40 μg) was dissolved in acetone (200 μL) to which 0.1 N hydrochloric acid (20 μL) was added. This solution was stirred at 25 °C for 24 h, after which the solvent was removed by a stream of nitrogen. FABMS indicated that a small amount of the acetonide **146** was formed: m/z 592, 452, 438, 326.3430, 300, 286, 268. Anal. Calcd. For $\text{C}_{21}\text{H}_{44}\text{NO}$: 326.3423 (M+H). Found 326.3430 (HRFABMS).

(4S, 5R)-4-Methyl-5-(n-pentadecyl)-oxazolidinone (158)

(2S, 3R)-2-amino-3-octadecanol (**155**, 750 μg , 2.6 μmol) was dissolved in dichloromethane (100 μL) to which 1, l'-carbonyldiimidazole (0.85 mg, 5.3 μmol) and triethylamine (0.4 μL , 2.9 μmol) was added. The solution was stirred for 5 h and then the solvent removed by a stream of nitrogen. The crude product **158** was analyzed without purification: IR (NaCl) 36, 2919, 2851, 1742, 1713, 1551, 1470, 1395, 1321, 49, 1239, 1094, 1061, 1001, 768, 743, 664 cm^{-1} ; FABMS m/z 785, 623, 474, 406, 362, 328, 312, 286, 268. Anal. Calcd. For $\text{C}_{19}\text{H}_{38}\text{NO}_2$: 312.2903 (M+H). Found: 312.2903 (HRFABMS).

Further Investigation of Changes in Cell Morphology

Materials

Lysophosphatidic acid (LPA), antibodies against tubulin and phalloidin were all obtained from Sigma. Fluorescein- and Texas red-labelled goat antimouse antibody were obtained from Amersham (U. K.). Antibody raised against the Rho protein was obtained from Sta Cruz Biotechn.

Cell culture

Vero cells were grown in Dulbecco's modified Eagle medium supplemented with 10% foetal bovine serum. Spisulosine or LPA were added to these cultures to a concentration of 0.2 - 1.0 mg and 50-10 mM respectively, from 4 to 24 hours. Cells were counted with the drug exclusion haemocytometer procedure using a solution of 0.4% Trypan blue in Hanks buffered Saline (Celis and Celis, "General Procedures for Tissue Culture in Cell Biology, a Laboratory Handbook" Academic Press Inc, Vol 1, pp. 5-17.)

Example A Spisulosine 285 causes changes in cell morphology

Vero cells were incubated with spisulosine 285 (0.5 mM) for 4 hours. Figure 3 is a microphotograph for the results in Example A. Cell shaped was altered from polygonal (untreated cells, panel a) to a fusiform shape (panel b). Panel c represents a higher magnification of the culture to which spisulosine was added.

Example B Change in cell morphology is due to an effect on the cell microfilaments

In order to identify the ~~organisation~~organization of the microfilament and microtubule ~~organisation~~organization in cells treated with spisulosine 285, cells were stained with phalloidin to detect actin polymers, and an antitubulin antibody to detect tubulin.

Vero cells were incubated in the presence (panel b, d) or absence (a, c) of 0.5 mM spisulosine 285 for 4 hours. Cells grown in coverslips were fixed with methanol at -20 °C (for tubulin antibody) or with 4 % paraformaldehyde in phosphate buffered saline PBS (w/v) for

phalloidin incubation. In the second case the cells were washed with 0.2% Triton X100 in PBS. The coverslips were washed with PBS and incubated for 1 hour at room temperature with the tubulin antibody (diluted 1/1000 in PBS) or with phalloidin (1 mg/ml). After washing with PBS the coverslips incubated with the tubulin antibody were overlaid with fluorescein or Texas red-labelled goat antimouse antibodies (diluted 1:50 in PBS). The coverslips were mounted with Mowiol and stored in the dark at 4 °C until observation.

Figure 4 is a microphotograph for the results in Example B. Panel 'a' represents cells stained with phalloidin (actin stain) and not treated with spisulosine. Panel 'b' represents cells stained for phalloidin and treated with spisulosine. Panel 'c' represents cells stained for tubulin and not treated with spisulosine. Panel 'd' represents cells stained for phalloidin and treated with spisulosine. There is a dramatic decrease in actin in spisulosine-treated cells, in comparison with untreated cells. Under the same conditions, the microtubule network remains in a polymerised form.

Example C Effect of spisulosine 285 on the Rho protein

The small GTP binding protein Rho is involved in the formation of actin-myosin "stress ~~fibres~~fibers" (Hall, A., Science, 279, 1998, p 509 - 514). Therefore, the electrophoretic mobility and cellular distribution of Rho was ~~analysed~~analyzed in cells treated with spisulosine 285.

Figure 5 is an electrophoretogram of Example C. In panel A, equivalent amounts of protein from a cell extract from untreated (a) or from 0.5 mM spisulosine 285 treated (20 hour) cells (b) were fractionated by gel electrophoresis and blotted onto nitrocellulose paper to ~~analyse~~analyze the amount of the Rho protein.

In panel B the experiment was carried out as above, except that the homogenate was fractionated into a particulate (membrane, "M") fraction and soluble ("S") fraction.

Subcellular fractionation was carried out by placing cells in a hypotonic buffer (0.25 M sucrose, 20mM HEPES pH 7.4, 2 mM EDTA, 1 mM PMSF, 10 mg/ml aprotinin, leupeptine and pepstatine), and lysing them with a Dounce. The homogenate was first centrifuged at 750 g for 5 minutes to remove nuclei and unbroken cells, and the supernatant was further centrifuged at 30,000 g for 1 hour (4 °C) to isolate a pelleted particulate fraction (putative membrane fraction) and a supernatant. The different fractions were characterised by electrophoresis and Western Blotting using an antibody against the Rho protein.

No significant change in the amount or mobility of Rho was observed on treatment of cells with spisulosine 285. However, a decrease in the proportion of Rho associated with the particulate fraction was observed.

Example D Effect of lysophosphatidic acid (LPA) on the action of spisulosine 285

LPA is known to increase the level of stress ~~fibres~~fibers in cells by activation of the Rho protein. The effect of LPA on cells treated with spisulosine 285, and untreated cells was examined.

Vero cells were incubated in the absence (a) or presence (b) of 10 mM LPA for 2 hours, or in the presence (c) of 0.5mM spisulosine 285 for 20 hours, or in the presence (d) of first 10mM LPA (2 hours) and afterwards with 0.5mM spisulosine 285 for an additional 18 hours.

Figure 6 is a microphotograph for the results in Example D. Panel b indicates the effect of LPA in increasing the level of actin. Incubation of Vero cells with spisulosine for 24 hours results in the appearance of rounded cells, see panel c. These cells detach from the culture dish and die. The addition of LPA prior to spisulosine prevents the morphological change promoted by spisulosine.

***in vivo* Data**

Example E -The effect of Spisulosine 285 *in vivo*

Spisulosine 285 was tested in *in vivo* studies against xenograft models of human prostate cancer (PC-3) and human renal cancer (MRI-H-121). These models use subcutaneously implanted solid human tumors that grow and increase in volume over time. The mean volume of tumor growth in control animals provides the basis for comparison. For active compounds the tumor growth is inhibited either completely (%T/C values< 1%, or negative), or partially(> 1%

T/C - 50% T/C). A level of activity that is less than 40% T/C is considered statistically significant. The doses of spisulosine used were given at the maximum tolerated, non lethal dose (MTD), 1/2 MTD and 1/4 MTD. Delivery of the drug was by the intraperitoneal route.

Human prostate cancer PC-3

Compound	Total Dose (mg/kg)	% T/C	Day	Comments
Spisulosine 285	9.990	-21%	11	stasis (complete remission)
Spisulosine 285	5.010	-1%	11	stasis (complete remission)
Spisulosine 285	2.499	223%	15	
Control		100%	15	

Human MRI-H-121 renal cancer

Compound	Total Dose (mg/kg)	% T/C	Day	Comments
Spisulosine 285	9.990	28%	11	inhibition (partial remission)
Spisulosine 285	5.010	35%	11	inhibition (partial remission)
Spisulosine 285	2.499	43%	15	
Control		100%	15	

Spisulosine 285 is effective against both tumour types, significantly reducing the tumour size in the case of the human prostate cancer model PC-3 at higher doses. Spisulosine 285 reduces the growth of the human renal cancer, with effects continuing up to a few weeks

after the last dose of the drug.

Example F

An expanded *in vitro* screen was performed of spisulosine 285 against a series of different cell lines. The following data was obtained:

Category	Line	Tumour <u>Tumor</u>	IC50	CV-1 Therapeutic Index
Solid	SK-HEP-1	Liver	3.51 E-15	7863
	PANC-1	Pancreas	1.71 E-12	16
	HT-29	Colon	2.56 E-12	11
	786-0	Renal	2.75 E-12	10
	FADU	Pharynx	4.99 E-12	6
	Hs 746T	Stomach	7.89 E-12	3
	SK-OV-3	Ovary	1.40 E-11	2
	MX-1	Mammary	3.89 E-11	1
	RAMOS	Burkitts	4.82 E-11	1
	P3HR1	Burkitts	6.73 E-11	0
	SW684	Fibrosarcoma	1.05 E-09	0
	U-937	Lymphoma	1.96 E-11	1
Lymphoma	H9	Lymphoma	3.10 E-11	1

Leukemia	HL60	Leukemia	8.5 0 E-12	3
	ARH77	Leukemia	1.36 E-12	2
	K562	Leukemia	1.57 E-11	2
	CCRF-SB	Leukemia	1.05 E-09	0
Normal	CV-1	Kidney fibroblasts	2.76 E-11	1

The range of IC₅₀ potencies against the ~~tumour~~tumor cell lines are from nanomolar, 1.05 E-09 nM, to ~~fentamolar~~femtomolar, 3.51 E-15 mM. It is exceptional to go beyond the nM and pM range to find a drug which has activity in the ~~mM~~ fM range.

The activities against the solid tumours were generally 1 log more potent than against the leukemias and lymphomas. Among the solid tumours, the most slow growing were the most sensitive, culminating with the very slow growing hepatoma SK-HEP-1.

The best therapeutic indices compared to the CV-1 normal cell line were seen with the slow growing solid tumors, since the IC₅₀ potency (2.76 E-11) was comparable to the leukemia/lymphomas. The solid tumor TIs ranged from 1- 20 units and the TI for the hepatoma was >3 log.

The renal tumour cell line was in the most active group, pM potencies, which correlates well to the *in vivo* xenograft data.

References

The following references provide background information related to the present invention. The disclosures of each are hereby incorporated herein by reference.

Faulkner, D. *J. Nat. Prod. Rep.* 1991, 8, 97-147.

Munro, M. H. G.; Luibrand, R. T.; Blunt, J. W. In *Bioorganic Marine Chemistry*; Scheuer, P. J., Ed.; Springer-Verlag: Berlin, 1987; Vol 1, pp. 93-176.

Bergmann, W.; Burke, D. C. *J. Org. Chem.* 1955, 20, 1501-1507.

Bergmann, W.; Burke, D. C. *J. Org. Chem.* 1956, 21, 226-228.

Bergmann, W.; Stempien, M. F., Jr. *J. Org. Chem.* 1957, 22, 1575-1577.

Rinehart, K. L., Jr.; Gloer, J. B.; Hughes, R. G., Jr.; Renis, H. E.; McGovren, J. P.; Swyenberg, E. B.; Stringfellow, D. A.; Kuentzel, S. L.; Li, L. H. *Science* 1981, 212, 933-935.

Rinehart, K. L.; Kishore, V. Bible, K. C.; Sakai, R.; Sullins, D. W.; Li, K.-M. *J. Nat. Prod.* 1988, 51, 1-21.

Rinehart, K. L.; Kishore, V.; Nagarajan, S.; Lake, R. J.; Gloer, J. B.; Bozich, F. A.; Li, K.-M.; Maleczka, R. E., Jr.; Todsén, W. L.; Munro, M. H. G.; Sullins, D. W.; Sakai, R. *J. Am. Chem.*

Soc. 1987, 109, 6846-6846.

Pettit, G. R.; Herald, C. L.; Doubek, D. L.; Herald, D. L., Arnold, E.; Clardy, J. *J. Am. Chem.*

Soc. 1982, 104, 6846-6848.

Kraft, A. S.; Smith, J. B.; Berkow, R. L. *Proc. Natl. Acad. Sci. USA* 1986, 83, 1334-1338.

Kupchan, S. M.; Britton, R. W.; Ziegler, M. F.; Sigel, C. W. *J. Org. Chem.* 1973, 38, 178-179.

Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* 1978, 43, 2923-2925.

Blunt, J. W.; Calder, V. L.; Fenwick, G. D.; Lake, R. J.; McCombs, J. D.; Munro, M. H. G.;

Perry, N. B. *J. Nat. Prod.* 1987, 50, 290-292.

Foucault, A. P. *Anal. Chem.* 1991, 63, 569A-579A.

McAlpine, J. B.; Hochlowski, J. E. In *Natural Products Isolation*; Wagman, G. H., Cooper, R.,

Eds.; *J. Chromatogr. Lib.* 43; Elsevier: New York, 1989, Chapter 1. Countercurrent

Chromatography: Theory and Practice; Mandava, N. B.; Ito, Y., Eds.; *Chromatography Science*
44; Dekker: New York, 1988.

Conway, W. D. *Countercurrent Chromatography: Apparatus, Theory, and Applications*; VCH:
New York, 1990.

Marston, A.; Slacanin, I.; Hostettmann, K. *Phytochem. Analysis* 1990, 1, 3-17.

Schaufelberger, D. E. *J. Chromatogr.* 1991, 538, 45-57.

Ito, Y. *J. Chromatogr.* 1981, 214, 122-1.

Bruening, R. C.; Oltz, E. M.; Furukawa, J.; Nakaninshi, K.; Kustin, K. *J. Nat. Prod.* 1986, 49, 193-204.

Pettit, G. R.; Gao, F.; Sengupta, D.; Coll, J. C.; Herald, C. L.; Doubek, D. L.; Schmidt, J. M.;
Van Camp, J. R.; Rudloe, J. J.; Nieman, R. A. *Tetrahedron* 1991, 47, 3601-3610.

Kohmoto, S.; McConnell, O. J.; Wright, A.; Cross, S. *Chem. Lett.* 1987, 1687-1690.

Martin, D. G. In *Countercurrent Chromatography: Theory and Practice*; Mandava, N. B.; Ito, Y., Eds.; Chromatography Science 44; Dekker: New York, 1988, Chapter 9.

Marayama, W.; Kobayashi, T.; Kosuge, Y.; Yano, H.; Nunogaki, Y.; Nunogake, K. *J. Chromatogr.* 1982, 239, 643-649.

Schaufelberger, D. E.; Chmurny, G. N.; Beutler, J. A.; Kolek, M. P.; Alvarado, A. B.;
Schaufelberger, B. W.; Muschik, G. M. *J. Org. Chem.* 1991, 56, 2895-2900.

Schaufelberger, D. E., Sandoz Pharma A G, personal communication, 1991.

Pettit, G. R.; Gao, F.; Herald, D. L.; Blumberg, P. M.; Lewin, N. E.; Neiman, R. A. *J. Am Chem. Soc.* 1991, 113, 6693-6695.

Kernan, M. R.; Molinski, T. F.; Faulkner, D. J. *J. Org. Chem.* 1988, 53, 5014-5020.

Murata, M.; Legrand, A. M.; Ishibashi, Y.; Fukani, M.; Yasumoto, T. *J. Am. Chem. Soc.* 1990, 112, 4380-4386.

Sakemi, S.; Ichiba, T.; Kohomoto, S.; Saucy, G.; Higa, T. *J. Am Chem. Soc.* 1988, 110, 4851-4853.

Sakemi, S.; Higa, T.; Anthoni, U.; Christophersen, C. *Tetrahedron* 1987, 43, 263-268.

Sun, H. H. Cross, S. S. Gunasekera, M.; Koehn, F. E. *Tetrahedron* 1991, 47, 1185-1190.

Kohomoto, S.; McConnell, O. J.; Wright, A.; Koehn, F.; Thompson, W.; Lui, M.; Snader, K. M. *J Nat. Prod.* 1987, 50, 336.

Ravi, B. N.; Perzanowski, H. P.; Ross, R. A.; Erdman, T. R.; Scheuer, P. J.; Finer, J.; Clardy, J. *Pure & Appl. Chem.* 1979, 51, 1893-1900.

Rinehart, K. L., Jr.; Sakai, R.; Stroh, J. G. U.S. Pat. No. 4,948,791, 1990; *Chem. Abs.* 1991, 114, 214413h.

Stierle, A. C.; Cardellina, J. H., II; Singleton, F. L. *Experientia* 1988, 44, 1021.

Schmitz, F. J.; Vanderah, D. J.; Hollenbeak, K. H.; Enwall, C. E. L.; Gopichand, Y.; SenGupta, P. K.; Hossain, M. B.; van der Helm, D. *J. Org. Chem.* 1983, 48, 3941-3945.

Dillman, R. L.; Cardellina, J. H., II *J. Nat. Prod.* 1991, 54, 1159-1161.

Stierle, A. C.; Cardellina, J. H., II; Strobel, G. A. *Proc. Natl. Acad. Sci. USA* 1988, 85, 8008-8011.

Stierle, A. A.; Cardellina, J. H., II; Singleton, F. L. *Tetrahedron Lett.* 1991, 32, 4847-4848.

Raub, M. F.; Cardellina, J. H., II; Choudhary, M. I.; Ni, C.-Z.; Clardy, J.; Alley, M. C. *J. Am. Chem. Soc.* 1991, 113, 3178-3180.

Sakemi, S.; Totton, L. E.; Sun, H. H. *J. Nat. Prod.* 1990, 53, 995-999.

Stierle, D. B.; Faulkner, D. J. *J. Nat. Prod.* 1991, 54, 1134-1136.

Kohomoto, S.; McConnell, O. J.; Wright, A. *Experientia* 1988, 44, 85-86.

Sun, H. H.; Sakemi, S. *J. Org. Chem.* 1991, 56, 4307-4308.

Dillman, R. L.; Cardellina, J. H., II *J. Nat. Prod.* 1991, 54, 1056-1061.

Sakai, R.; Kohmoto, S.; Higa, T.; Jefford, C. W.; Bernardinelli, G. *Tetrahedron Lett.* 1987, 28, 5493-5496.

Bobzin, S. C.; Faulkner, D. J. *J. Org. Chem.* 1991, 56, 4403-4407.

Sakemi, S.; Sun, H. H. *J. Org. Chem.* 1991, 56, 4304-4307.

Keifer, P. A.; Schwartz, R. E.; Koker, M. E. S.; Hughes, R. G., Jr.; Rittschoff, D.; Rinehart, K. L. *J. Org. Chem.* 1991, 56, 2965-2975.

Schaufelberger, D. E.; Pettit, G. R. *J. Liq. Chromatogr.* 1989, 12, 1909-1917.

Pettit, G. R.; Herald, C. L.; Leet, J. E.; Gupta, R.; Schaufelberger, D. E. Bates, R. B.; Clelow, P. J.; Doubek, D. L.; Manfredi, K. P.; Rutzler, K.; Schmidt, J. M.; Tackett, L. P.; Ward, F. B.; Bruck, M.; Camou, F. *Can. J. Chem.* 1990, 68, 1621-1624.

Schmitz, F. J.; DeGuzman, F. S.; Choi, Y.-H.; Hossain, M. B.; Rizvi, S. K.; van der Helm, D.

Pure & Appl. Chem. 1990, 62, 1393-1396.

Schmitz, F. J.; DeGuzman, F. S.; Hossain, M. B.; van der Helm, D. *J. Org. Chem.*, 1991, 56, 804-808.

Gunawardana, G. P.; Kohmoto, S.; Gunasekera, S. P.; McConnell, O. J.; Koehn, F. E. *J. Am. Chem. Soc.* 1988, 110, 4856-4858.

Gunawardana, G. P.; Koehn, F. E.; Lee, A. Y.; Clardy, J.; He, H.-y.; Faulkner, D. J. *J. Org. Chem.* 1992, 57, 1523-1526.

Gunawardana, F. P.; Kohmoto, S.; Burres, N. S. *Tetrahedron Lett.* 1989, 30, 4359-4362.

Sakemi, S.; Sun, H. H.; Jefford, C. W.; Bernardinelli, G. *Tetrahedron Lett.* 1989, 30, 2517-2520.

Sun, H. H.; Sakemi, S.; Burres, N.; McCarthy, P. *J. Org. Chem.* 1990, 55, 4964-4966.

Perry, N. B.; Blunt, J. W.; Munro, M. H. G.; Higa, T.; Sakai, R. *J. Org. Chem.* 1988, 53, 43-44.

Jares-Erijman, E. A.; Sakai, R.; Rinehart, K. L. *J. Org. Chem.* 1991, 56, 5712-5715.

Berlinck, R. G. S.; Braekman, J. C.; Daloze, D.; Hallenga, K.; Ottinger, R.; Bruno, I.; Riccio, R. *Tetrahedron Lett.* 1990, 31, 6531-6534.

Kashman, Y.; Hirsh, S.; McConnell, O. J.; Ohtani, I.; Kusumi, T.; Kakisawa, H. *J. Am. Chem. Soc.* 1989, 111, 8925-8926.

Rinehart, K. L.; Holt, T. G.; Fregeau, N. L. Stroh, J. G.; Keifer, P. A.; Sun, F.; Li, L. H.; Martin, D. G. *J. Org. Chem.* 1990, 55, 4512-4515.

Wright, A. E.; Forleo, D. A.; Gunawardana, G. P.; Gunasekera, S. P.; Koehn, F. E.; McConnell, O. J. *J. Org. Chem.* 1990, 55, 4508-4512.

Vaught, K. C. A. *Classification of the Living Mollusca*; Abbott, R. T.; Boss, K. J., Eds.; American Malacologists: Melbourne, Fla. 1989; pp. 113-6.

Brusca, R. C.; Brusca, G. J. *Invertebrates*; Sinauer Associates: Sunderland, Mass., 1990; pp. 706-709.

Barnes, R. D. *Invertebrate Zoology*; 4.sup.th ed.; Saunders College: Philadelphia, 1980; p 425.

Engemann, J. G.; Hegner, R. W. *Invertebrate Zoology*; 3rd ed.; Macmillan: New York, 1981; pp. 454-467.

Palameta, B.; Proštenik, M. *Croat. Chem. Acta* 1961, 33, 133-135.

Kawano, Y.; Highchi, R.; Isobe, R.; Komori, T. *Liebigs Ann. Chem.* 1988, 19-24.

Gulavita, N. K.; Scheuer, P. J. *J. Org. Chem.* 1989, 54, 366-369.

Jimenez, C.; Crews, P. *J. Nat. Prod.* 1990, 53, 978-982.

Mori, K.; Matsuda, H. *Liebigs Ann. Chem.* 1992, 131-137.

Proštenik, M.; Alaupovic, P. *Croat. Chem. Acta* 1957, 29, 393-402.

Osby, J. O.; Martin, M. G.; Ganem, B. *Tetrahedron Lett.* 1984, 25, 2093-2096.

Stoffel, W. *Ann. Rev. Biochem.* 1971, 40, 57-82.

Merrill, A. H., Jr.; Nimkar, S.; Menaldino, D.; Hannun, Y. A.; Loomis, C.; Bell, R. M., Tyagi, S.
R.; Lambeth, J. D.; Stevens, V. L.; Hunter, R.; Liotta, D. C. *Biochemistry* 1989, 28, 3138-3145.

Hannun, Y. A.; Bell, R. B. *Science* 1989, 243, 500-507.

Merrill, A. H., Jr. *J. Bioenerg. Biomem.* 1991, 23, 83-104.

Witten, J. L.; Schaffer, M. H.; O'Shea, M.; Cook, J. C.; Hemling, M. E.; Rinehart, K. L., Jr.
Biochem. Biophys. Res. Commun. 1984, 124, 350-358.

Shaw, P. D.; McClure, W. D.; Van Blaricom, F.; Sims, J.; Fenical, W.; Rude, J. In *Food and Drugs from the Sea 1974*; Webber, H. H.; Ruggieri, G. D., Eds.; Marine Technological Society: Washington, D. C., 1976; pp. 429-433.

Herrmann, E. C., Jr. *Progr. Med. Virol.* 1961, 3, 158-192.

Grode, S. H.; Cardellina, J. H., II *Lipids*, 1983, 18, 889-893.

Mackay and Hall, *J. Biol. Chem.* 273, 20685-20688, 1998).

Hall, A., *Science* 279, 509-514, 1998

Itoh, et al., *Nature Medicine*, Vol 5, No. 2, 1999

Celis and Celis, *General Procedures for Tissue Culture in Cell Biology, a Laboratory Handbook* Academic Press Inc, Vol 1, pp. 5-17.)

The present invention has been described in detail, including the preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of the present disclosure, may make modifications and/or improvements on this invention.

This invention was made with Government support under Contract Number AI-04769
awarded by the National Institutes of Health (NIH). The Government has certain rights in the
invention.